



Heparins and Fucoidans: Future Therapeutic Options for Ulcerative Colitis

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Table of Contents

Declaration of Originality.....	i
Authority of Access.....	i
Statement of Ethical Conduct.....	i
Statement regarding published work contained in thesis	ii
List of Publications and Statement of Co-authorship.....	ii
Acknowledgements	vi
List of Abbreviations	xi
List of Tables	xiii
List of Figures.....	xiv
Abstract.....	xvi
Chapter 1 Literature Review.....	1
1.1 Ulcerative colitis.....	1
1.1.1 Pathophysiology of UC	2
1.1.2 Current management strategies	7
1.2 Heparins.....	9
1.2.1 Potential mechanism of actions of heparins in UC	11
1.2.2 Pre-clinical studies of heparins in UC	18
1.2.3 Clinical use of heparins in UC.....	21
1.2.4 Proposed reasons for reported inconsistent clinical outcomes of heparins in UC	28
1.2.5 Complexity of heparins	33
1.3 Fucoidans.....	35
1.3.1 Fucoidans in inflammation	37
1.3.2 Fucoidans in UC	38
1.3.3 Prospects and challenges in the development of fucoidan-based therapy in UC	40
1.4 Rationale of study	43
1.5 Aim and objectives	45
Chapter 2 Identification of Pro- and Anti-proliferative Oligosaccharides of Heparins	46
2 Abstract.....	46
2.1 Introduction	47
2.2 Materials and methods.....	49

2.2.1	Foetal calf serum mitogenic assay	50
2.2.2	Basic fibroblast growth factor mitogenic assay	50
2.2.3	Quantification of cell proliferation using WST-1 reagent	51
2.2.4	UFH and LMWHs	51
2.2.5	Isolation of enoxaparin oligosaccharides	51
2.2.6	Cell growth assay	53
2.2.7	Protein quantification	54
2.2.8	Colony formation assay	54
2.2.9	Cell viability assays	55
2.2.10	Cell cycle analysis	57
2.2.11	Statistical analysis	58
2.3	Results	58
2.3.1	Serum-dependent cell growth	58
2.3.2	Effect of basic fibroblast growth factor on HCT-116 cells	59
2.3.3	Serum dependency of the effect of heparins on cell growth	62
2.3.4	Dose-dependent reduction of colony formation by heparins	64
2.3.5	Inhibition of cell cycle progression by heparins	66
2.3.6	Effect of heparins on cellular viability	67
2.3.7	Differential effects of enoxaparin oligosaccharides on proliferation and Cell cycle	69
2.4	Discussion	72
2.5	Conclusion	78
Chapter 3 Orally Administered Enoxaparin Ameliorates Acute Colitis by Reducing Macrophage-associated Inflammatory Responses		80
3	Abstract	80
3.1	Introduction	81
3.2	Materials and methods	84
3.2.2	Formulation of enoxaparin	84
3.2.3	Treatment with enoxaparin	85
3.2.4	Evaluation of intestinal inflammation	85
3.2.5	Termination of experiment and tissues sampling	86
3.2.6	Histologic evaluation of colitis	86
3.2.7	Immunohistochemistry	86

3.2.8 Tissue explant culture and measurement of cytokine levels	88
3.2.9 Statistical analysis	89
3.3.1 Orally administered enoxaparin ameliorates colitis-induced weight loss	89
3.3.2 Enoxaparin ameliorates colitis-associated diarrhoea and blood in stool.....	89
3.3.3 Orally administered enoxaparin alleviates macroscopic changes of the colon	91
3.3.4 Orally administered enoxaparin decreases colon damage and infiltration of inflammatory cells.....	93
3.3.5 Orally administered enoxaparin reduces the levels of pro-inflammatory cytokines	95
3.3.6 Orally administered enoxaparin affects macrophage numbers and differentiation status during colitis.....	100
3.3.7 Orally administered enoxaparin retains occludin and claudin-4 by attenuating crypt loss.....	105
3.4 Discussion.....	107
3.5 Conclusion.....	112
Chapter 4 Fucoidan Extracts Ameliorate Acute Colitis	113
4 Abstract.....	113
4.1 Introduction	115
4.2 Materials and methods.....	117
4.2.1 Animal colitis model	117
4.2.2 Formulation of fucoidan extracts.....	118
4.2.3 Treatment with fucoidan extracts	119
4.2.4 Evaluation of intestinal inflammation	119
4.2.5 Termination of experiment and tissue sampling	119
4.2.6 Histologic evaluation of colitis.....	120
4.2.7 Tissue explant culture and measurement of cytokine levels	120
4.2.8 Statistical analysis	121
4.3 Results	121
4.3.1 Oral fucoidan extracts ameliorate colitis-induced weight loss.....	121
4.3.2 Oral fucoidan extracts delay the development of diarrhoea and the appearance of blood in stool.....	123
4.3.3 Oral fucoidan extracts protect against changes to colon and spleen	123

4.3.4	Oral fucoidan extracts decrease colon damage and infiltration of inflammatory cells.....	126
4.3.5	Oral fucoidan extracts reduce the levels of pro-inflammatory cytokines	128
4.4	Discussion.....	133
4.5	Conclusion.....	137
Chapter 5 Non-Anticoagulant Oligosaccharides of Enoxaparin Relieve Chemical		
	-induced Acute Colitis	139
5	Abstract.....	139
5.1	Introduction	140
5.2	Materials and methods.....	141
5.2.1	Preparation of oligosaccharides.....	141
5.2.2	Formulation of enoxaparin oligosaccharides for <i>in vivo</i> administration	143
5.2.3	Animal colitis model	144
5.2.4	Treatment with oligosaccharides	145
5.2.5	Evaluation of colitis disease activity	146
5.2.6	Termination of experiments and sampling of colon.....	147
5.2.7	Statistical analysis	147
5.3	Results	147
5.3.1	Separation, isolation and desalting of enoxaparin oligosaccharide.....	147
5.3.2	Induction of acute colitis with 3% of w/v DSS	149
5.3.3	Effects of PFs of enoxaparin in acute colitis	150
5.3.4	Induction of acute colitis with 5% of w/v DSS	153
5.3.5	Effects of sub-fractions of enoxaparin in acute colitis	154
5.4	Discussion.....	157
5.5	Conclusion.....	160
Chapter 6 General Conclusion.....		
6.1	Summary.....	162
6.2	Limitations.....	165
6.3	Future directions	166
Chapter 7 References.....		
Chapter 8 Appendices.....		

List of Abbreviations

bFGF	basic fibroblast growth factor
CD	Crohn's disease
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DNBS	di-nitrobenzenesulfonic acid
Dp	saccharide units
DSS	dextran sulphate sodium
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
GAGs	glycosaminoglycans
G-CSF	granulocyte colony-stimulating factor
GIT	gastrointestinal tract
GM-CSF	granulocyte-macrophage colony-stimulating factor
H&E	hematoxylin and eosin
H ₂ O ₂	hydrogen peroxide
HS	heparan sulphate
IBD	inflammatory bowel disease
IC	ion-exchange chromatography
ICAM	intercellular adhesion molecule
IL	interleukin
iNOS	inducible nitric oxide synthase
LMWF	low molecular weight fucoidan
LMWH	low molecular weight heparin
LPS	lipopolysaccharide
MEM	minimal essential medium
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
MPO	myeloperoxidase
MW	molecular weight
NF-κB	nuclear-factor kappa B

NaCl	sodium chloride
NK T	natural killer T cell
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
PBS	phosphate buffered saline
PF	pooled fraction
PI	propidium iodide
PSGL-1	P-selectin glycoprotein ligand-1
RPMI	Roswell Park Memorial Institute
SD	standard deviation
TBST	Tris-buffered saline containing 0.5% v/v Tween 20
TCR	T cell receptor
Treg	T regulatory cell
TGF	transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNBS	tri-nitrobenzene sulfonic acid
TNF	tumour necrosis factor
UC	ulcerative colitis
UFH	unfractionated heparin
VCAM-1	vascular cell adhesion molecule-1
WST	water soluble tetrazolium

List of Tables

Table 1.1 Use of heparins in murine experimental colitis.	20 - 21
Table 1.2 Use of heparins in treating patients with UC.....	25 - 27
Table 1.3 Different LMWHs with their methods of depolymerisation, average molecular weight and anticoagulant activity.	31
Table 1.4 Fucoidans in murine experimental colitis.....	40
Table 3.1 Scoring system for stool consistency and blood in stool.....	85
Table 4.1 Fucoidan extract composition	118
Table 4.2 Effect of fucoidan extracts on colon-derived cytokine levels.	130
Table 5.1 Composition of food mash for oral treatment in mice.	143
Table 5.2 Percentage (%) and dose of pooled fractions and sub-fractions of enoxaparin.	146
Table 5.3 Scoring system for stool consistency and blood in stool.....	146

List of Figures

Figure 1.1 Immunopathogenic mechanisms of UC.	3
Figure 1.2 Structure of heparins.	11
Figure 1.3 Potential heparin-mediated mechanisms in UC.	12
Figure 1.4 Structure of fucoidans.	36
Figure 2.1 FCS-dependent epithelial cell growth.	59
Figure 2.2 Epithelial cell growth with bFGF.	61
Figure 2.3 Effect of heparins on epithelial cell growth.	63
Figure 2.4 Inhibition cellular colony formation by heparins.	65
Figure 2.5 Inhibition of cell cycle progression by heparins.	66
Figure 2.6 Effect of heparins on cellular viability.	68
Figure 2.7 Differential effects of enoxaparin oligosaccharides on proliferation and cell cycle.	71
Figure 3.1 Effect of enoxaparin during acute colitis.	90
Figure 3.2.1 Representative images of gross appearance of cecum	91
Figure 3.2.2 Effect of enoxaparin on macroscopic appearance of colon.	92
Figure 3.3 Effect of enoxaparin on histological changes of colon.	94
Figure 3.4 Effect of enoxaparin on histological changes of colon.	95
Figure 3.5.1 Effect of enoxaparin on colonic cytokine levels.	96
Figure 3.5.2 Effect of enoxaparin on colonic cytokine levels.	97
Figure 3.6 Correlation between colonic cytokine levels and body weight changes during acute colitis.	99
Figure 3.7 Effect of enoxaparin on macrophage infiltration into the inflamed colon.	101
Figure 3.8 Effect of enoxaparin on macrophage phenotypes.	103
Figure 3.9 Effect of enoxaparin on macrophage phenotypes.	104
Figure 3.10 Effect of enoxaparin on IL-1 β expression on colon tissues.	105
Figure 3.11 Effect of enoxaparin on epithelial tight junctions of colon.	106
Figure 4.1 Effect of fucoidan extracts during acute colitis.	122
Figure 4.2 Effect of fucoidan extracts on colon and spleen.	125
Figure 4.3 Effect of fucoidan extracts on colon histology.	127
Figure 4.4 Effect of fucoidan extracts on colon-derived cytokine levels.	129
Figure 4.5 Correlation between colon-derived cytokine levels and treatment-induced changes to body weight.	132

Figure 5.1 Schematic representation of the timeline of the acute colitis mouse model. ...	144
Figure 5.2 Experimental design of different groups of mice with and without treatment.	145
Figure 5.3 Ion-exchange chromatographic separation of enoxaparin and its oligosaccharides.....	149
Figure 5.4 Effects of enoxaparin oligosaccharides in DSS (3% w/v)-induced acute colitis.	152
Figure 5.5 Severity of acute colitis in 5% w/v DSS-treated C57BL/6 mice.	154
Figure 5.6 Effects of Effects of enoxaparin oligosaccharides in DSS (5% w/v)-induced acute colitis.	156

Abstract

Ulcerative colitis (UC) is a chronic inflammation of colonic mucosa and submucosa, characterised by abdominal pain, rectal bleeding and diarrhoea. Although the aetiology of UC remains elusive, the mucosal immune response, microbial features and intestinal epithelial barrier defects are all implicated in the pathogenesis of UC. Current drug therapies are not completely effective for the management of UC and many patients eventually require surgical interventions. Therefore there is a need for effective and safe therapies in UC.

Both heparins (unfractionated heparin, UFH and low molecular weight heparins, LMWHs) and heparin-like molecules such as fucoidans are complex mixture of polysaccharides. Heparins are widely used anticoagulants whereas fucoidan extracts are available as health supplements. Heparins and fucoidans have been reported to possess a wide range of biological activities including anti-inflammatory activity. At present, the use of heparins for the treatment of UC remains uncertain and requires further investigation. Moreover, the use of heparins is largely limited by the risk of bleeding in conditions where anticoagulation is not required. Therefore we aimed to investigate the therapeutic potential of sulphated polysaccharides (enoxaparin and fucoidans) in a model of colitis with a goal of identifying specific oligosaccharides that are responsible for their anti-inflammatory activity.

We first examined whether UFH, LMWHs (dalteparin and enoxaparin) and individual oligosaccharides of enoxaparin can affect cell growth *in vitro*. In UC, cell proliferation is required for mucosal healing. Enoxaparin was separated into different oligosaccharides by ion-exchange chromatography (IC). The proliferation and viability of human colonic epithelial cancer cells (HT-29, DLD-1 and HCT-116) were investigated in the presence and absence of different concentrations of UFH, dalteparin, enoxaparin and its

oligosaccharides. Protein quantification, the numbers of cell colonies and proportion of cells in different phases of the cell cycle were measured. Cell viability was assessed by a colorimetric method, trypan blue exclusion and by measuring apoptosis. Subsequently, investigation of the potential of fucoidan extracts, enoxaparin and its oligosaccharides in colitis was evaluated *in vivo*. Colitis was induced in male C57BL/6 mice by the administration of dextran sulphate sodium (DSS) through the drinking water. Mice received different treatment once daily either intraperitoneally or orally. Animals were monitored daily for their body weight, stool consistency and rectal bleeding. The experiment was terminated on day 8 and the colons of mice were weighed and processed for histology and to measure cytokine levels.

UFH, dalteparin and enoxaparin inhibited cell proliferation in a dose-dependent manner. This inhibitory effect was well-correlated with the induction of cell cycle arrest in the G1 phase and was not associated with any significant changes in cell viability. The isolated oligosaccharides of intact enoxaparin have different degrees of polymerisation ranging from 2 saccharides (dp2; ~ 600 Da) to 24 saccharides (dp24; ~ 8000 Da). Each IC-derived oligosaccharide of enoxaparin had either no, anti- or pro- proliferative effects, depending on its composition. Disaccharides devoid of anticoagulant activity had the strongest anti-proliferative effect, whereas the hexasaccharides promoted cell growth. *In vivo* results showed that orally administered but not intraperitoneal injected enoxaparin was effective in suppressing the pathology of colitis. The observed effect of oral enoxaparin corresponded well to the reduction of macrophage-associated responses as well as the suppression of inflammatory cytokine levels. Like enoxaparin, fucoidan extracts also ameliorated colitis by reducing the levels of a number of pro-inflammatory cytokines. Intriguingly, among oligosaccharides of enoxaparin tested, oligosaccharides shorter than dp8 were identified to be responsible for the anti-inflammatory activity of enoxaparin.

Tetrasaccharides and hexasacchrides were the two main active components that relieved colitis-associated body weight loss and prevented macroscopic changes to the colon tissue.

In conclusion, enoxaparin and fucoidan extracts possess anti-inflammatory activity and are effective in an acute colitis model. The *in vitro* and *in vivo* results highlighted that depending on their composition, different oligosaccharides can have distinctive effects. It is important to isolate and test different oligosaccharides from the mixture. This approach potentially leads to the identification of specific oligosaccharides that are responsible for anti-inflammatory effects of the parent molecules and could reduce the unwanted side effect associated with the administration of a mixture of polysaccharides. For example, the tetrasaccharide of enoxaparin, shorter than antithrombin-binding pentasaccharide, does not bind to antithrombin, thus its use is unlikely to be associated with a risk of bleeding even when administered at high doses. The identified non-anticoagulant enoxaparin oligosaccharides that have colitis-suppressing properties could therefore represent a novel therapeutic option for the management of UC.

Chapter 1

Chapter 1 Literature Review

1.1 Ulcerative colitis

Ulcerative colitis (UC) and Crohn's disease (CD) are the two main forms of inflammatory bowel diseases (IBD). Both are chronic, progressive inflammatory disorders of the gastrointestinal tract (GIT) [1]. UC is restricted to the submucosa or mucosa of the large intestine [2]. In UC, inflammation involves the rectum (proctitis), and may extend contiguously to the sigmoid colon (proctosigmoiditis), distal colon (left-sided colitis), transverse colon (extensive colitis) or the entire colon (pancolitis) [2]. Unlike in UC, patients with CD typically display a deeper transmucosal inflammation of any part of the GIT. However, CD is usually confined to the terminal portion of the small intestine, the ileum and colon [3]. Both types of IBD involve alterations of mucosal immunity and physiological responses. Even though UC and CD share several similar chronic and relapsing inflammatory characteristics, they differ in their pathological and clinical features, as well as in their responsiveness to certain therapeutic drugs [4].

Previously, northern Europe and North America were known as high-incidence and prevalence areas of UC. In 2004, more than 1 million people in Europe and 0.7 million people in the United States suffered from UC [5]. Recently, the number of UC cases has increased in countries where it was previously uncommon; this includes southern Europe, Australia, Africa, Latin America and Asia [5-7]. The rising incidence of this debilitating chronic inflammatory disease reinforces the need for a better understanding of the underlying pathogenesis, a prerequisite for the development of novel therapeutic approaches. A better insight into the medical management of UC ultimately improves patients' quality of life and reduces the risk of further complications.

1.1.1 Pathophysiology of UC

Although the full aetiology of UC remains unknown, it is clearly a multifactorial intestinal disease [1]. The intestinal barrier is constituted of epithelial cells that are tightly arranged and organised to control the permeability of luminal antigens. However, in UC, the integrity and function of the intestinal barrier is compromised due to a dysfunctional mucus layer, alterations of tight junction proteins and the presence of extracellular degrading enzymes [8,9]. As a result, luminal antigens have access into the mucosa across the disrupted epithelial barrier [9]. Excessive recognition and processing of microbial antigens and the release of pro-inflammatory mediators by antigen presenting cells, further enhances the migration and accumulation of inflammatory cells from the vasculature into the gut mucosa [10]. These activated innate immune and effector cells then release pro-inflammatory cytokines, perpetuating the cycle of chronic inflammation [2].

1.1.1.1 Epithelial barrier dysfunction

The intestinal epithelium serves as an important part of the host's immune defence by acting as a barrier to prevent bacterial entry into the lamina propria but selectively allows the passage of essential dietary nutrients, electrolytes and water into the systemic circulation [11]. A mucus layer, produced by goblet cells, permits the diffusion of required macromolecular components across the intestinal epithelium but prevents the entry of microbes from the gut to the lamina propria [12]. The mucus barrier contains secreted mucins, trefoil peptides, antimicrobial peptides and immunoglobulin A that contribute towards maintaining a balanced microbiota homeostasis and immune defence [12]. In UC, epithelial barrier dysfunction occurs as a result of diminished mucus secretion and defects at the level of epithelial tight junctions (Fig. 1.1) [8]. As the thickness and continuity of the mucus barrier is reduced and becomes less effective, bacteria are in direct contact with

epithelial cells [8]. With defective tight junction between the intestinal epithelial cells, increased permeability allows microbes or microbial antigens to translocate from the gut lumen to the lamina propria (Fig. 1.1) [13]. These bacterial antigens are subsequently recognised by antigen presenting cells, mainly macrophages and dendritic cells [10].

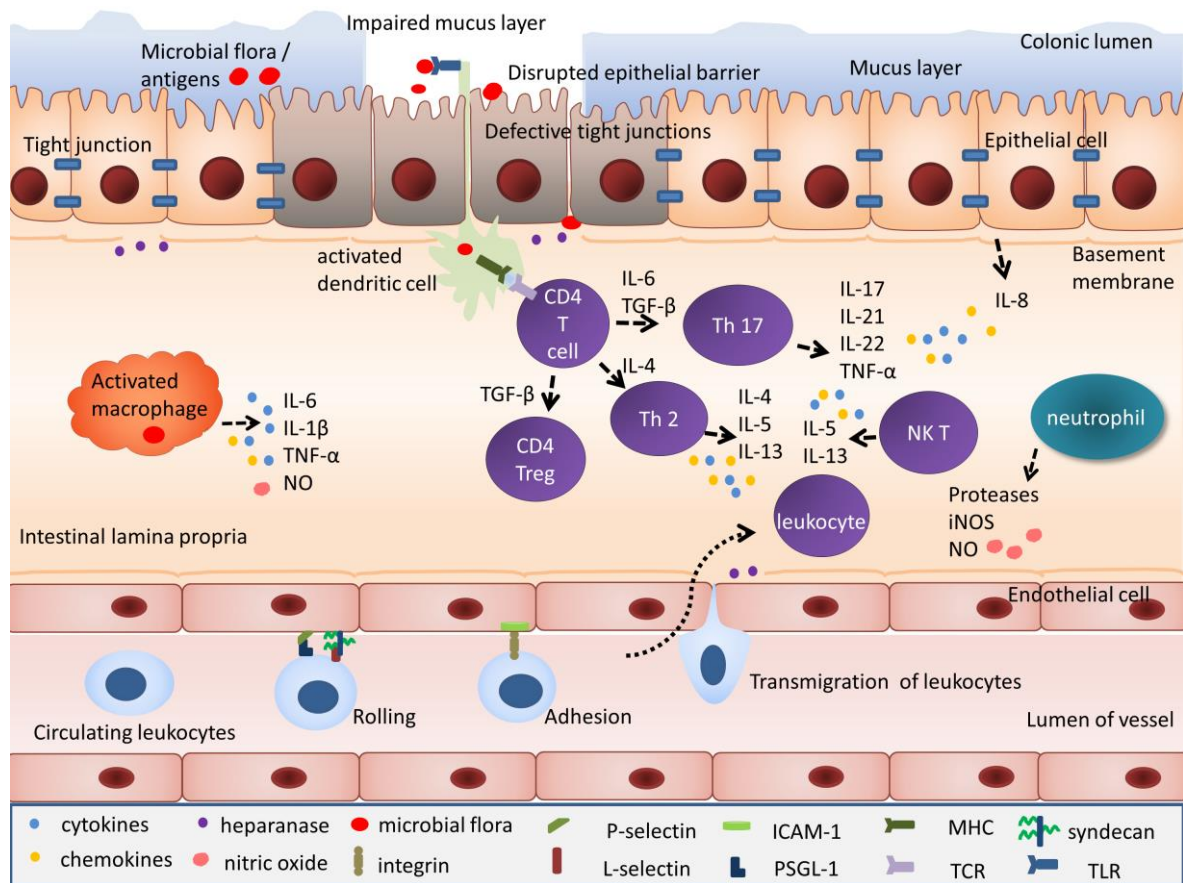


Figure 1.1 Immunopathogenic mechanisms of UC.

In UC, luminal antigens gain access to the intestinal lamina propria through disrupted epithelial barrier. The presence of pathogenic bacteria are detected by macrophages and dendritic cells through Toll-like receptors. Upon antigen recognition, activated macrophages and dendritic cells produce pro-inflammatory cytokines. Pro-inflammatory cytokines promote differentiation of naive T cells to effector T cells and further amplify the inflammatory immune response. Secreted pro-inflammatory mediators perpetuate the cycle of chronic inflammation by recruiting a large number of leukocytes including neutrophils and macrophages, from the circulation into the intestinal lamina propria. Interleukin, IL; inducible nitric oxide synthase, iNOS; intercellular adhesion molecule-1, ICAM-1; major histocompatibility complex, MHC; natural killer T cell, NK T; nitric oxide, NO; P-selectin

glycoprotein ligand-1, PSGL-1; T cell receptor, TCR; T helper cell, Th; Toll-like receptor, TLR; T regulatory cell, Treg; transforming growth factor- β , TGF- β ; tumour necrosis factor- α , TNF- α .

1.1.1.2 Antigen recognition

Luminal antigens are recognised by macrophages via Toll-like receptors (TLRs) during phagocytosis. Luminal antigens may also be sampled by activated dendritic cells, whose dendrites penetrate into the gut lumen via the paracellular space between epithelial cells (Fig. 1.1) [2]. In UC, dendritic cells accumulate at the site of inflammation and have exaggerated responses to antigenic stimuli [2]. Apart from macrophages and dendritic cells, intestinal epithelial cells also serve as antigen-presenting cells and play a key role in intestinal inflammation [14]. Under physiological conditions, intestinal epithelial cells express various types of pattern-recognition receptors such as TLRs and nucleotide oligomerization domain (NOD)-like receptors that recognise microbial antigens and are important in maintaining mucosal homeostasis [14]. However, in UC, increased expression of enterocyte TLRs is likely to trigger excessive immune responses against pathogens that invade the epithelial barrier. Activation of TLRs on epithelial cells by pathogen-associated molecular patterns induces a signalling cascade that results in the activation of nuclear-factor kappa B (NF- κ B) which stimulates the transcription of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, interferon (IFN)- γ and IL-1 β [4]. These pro-inflammatory mediators subsequently activate more target cells to mediate additional immune responses [15] as well as modify the function of tight junctions, which further increase epithelial permeability [13].

1.1.1.3 Dysregulation of mucosal immune response

Increased bacterial translocation and uptake clearly disturb intestinal homeostasis [10]. Neutrophils, macrophages and dendritic cells are important innate immune cells in the

maintenance of intestinal immune system homeostasis. However, excessive antigen recognition and aberrant immune responses lead to a massive recruitment and accumulation of these immune cells in the inflamed mucosa of patients with UC [1]. Activated neutrophils release pro-inflammatory cytokines and chemokines to attract monocytes and dendritic cells. At the same time, they also produce large amounts of reactive oxygen species that are intended to fight pathogens but which also damage the surrounding tissues [16]. Recruited macrophages produce significant amounts of TNF- α , which expands the inflammatory response [4]. When the excessive pathogenic challenge exceeds the killing capacity of macrophages and neutrophils, an adaptive immune response, which is initiated by dendritic cells, is predominantly involved.

Dendritic cells present antigens through the major histocompatibility complex class II (MHC-II) on the cell surface to naive T cells [4]. Activated naive T cells differentiate into different effector T cells and regulatory T cells (Treg) (Fig. 1.1) [10]. Helper T cells (Th1, Th2 and Th17) are critical for a protective immune response against pathogens. However, their exaggerated activation can generate and sustain the chronic inflammation observed in UC (Fig. 1.1) [10]. Th2 are the dominant T helper subset in UC, that produce IL-4, IL-5 and IL-13 [2,17]. Data from genome-wide association studies suggest that Th17 cells are also associated with UC [18]. These cells are activated by the transforming growth factor beta (TGF- β) + IL-6/ IL-23 pathway and secrete a number of pro-inflammatory cytokines including IL-17A, IL-21, IL-22 and IFN- γ . In particular IL-17A, which is upregulated in intestinal biopsies of UC patients, promotes neutrophil recruitment from blood vessels to the mucosa and induces IL-8 to be released from epithelial cells [18]. In patients with UC, the numbers of both peripheral blood and colonic Treg cells, which under normal conditions balance mucosal homeostasis, are decreased [19]. It is thought that this reduced number of Treg cells in UC is responsible for directing the active immune responses towards

inflammation [4]. Moreover, a high number of non-classical natural killer T (NK T) cells are detected in the lamina propria of the inflamed colon [20]. NK T cells are a major source of IL-13 and are associated with the lesions in UC [20]. IL-13 induces epithelial cell death, which further interrupts the integrity of the epithelial barrier and hence facilitates the infiltration of even more pathogens [20,21]. Additionally, IL-13-mediated epithelial cell damage induces the production of auto-antibodies against goblet cell glycoproteins, epithelial cells and other tissues that carry the same epitopes. Binding of auto-antibodies to endogenous glycoproteins may lead to further damage of the intestinal epithelium and manifestation of extra-intestinal inflammation observed in UC [22].

1.1.1.4 Leukocyte recruitment

Infiltration of leukocytes into the affected mucosa is a critical event during colitis. Adhesion molecules that include E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are required for leukocyte adhesion on the vascular endothelial cells before leukocytes can migrate into the gut mucosa. Upregulation of cell adhesion molecules enhances the recruitment of leukocytes [23]. Bacterial antigens and pro-inflammatory cytokines such as TNF- α , IL-1 β or lipopolysaccharide (LPS) have been shown to cause a pronounced upregulation of adhesion molecule expression on the surface of endothelial cells that increase leukocyte recruitment [24,25]. Imperatively, human intestinal microvascular endothelial cells from chronically inflamed tissue in patients with UC exhibit higher cytokine-mediated leukocyte-binding capacity when compared with endothelial cells derived from normal mucosa of uninvolved intestinal segments from the same patients [26]. Overall, this enhanced binding of leukocytes onto the endothelium facilitates the transmigration of immune cells from the vasculature into the intestinal lamina propria, which also contributes to chronic intestinal inflammation (Fig. 1.1) [23,24,27].

1.1.1.5 Heparanase as extracellular degrading enzyme

Heparanase is an endo- β -D-glucuronidase capable of degrading heparan sulphate (HS) chains [9]. HS chains are attached to the surface of many types of cells and extracellular matrix (ECM) proteins to form HS proteoglycans that play important functional and structural roles [9]. Specifically, in the gut, HS chains are found on epithelial cell surface, in the basal lamina of the intestinal mucosa as well as basement membrane surrounding the endothelium in the intestinal lamina propria [28]. Therefore they are involved in maintaining a healthy and functional gut barrier, regulating vascular permeability and facilitating cell-cell interactions [9,29]. Heparanase expression is reported to be higher in the inflamed mucosa of patients with chronic UC compared with people without UC [30,31]. It has been proposed that the presence of heparanase disintegrates HS within the basement membrane surrounding the endothelium in the intestinal lamina propria, which facilitates the migration of inflammatory cells from the circulation to the inflamed mucosa (Fig. 1.1). Heparanase also may cause substantial degradation of HS in the sub-epithelial basal lamina of the colon, contributing the loss of epithelial barrier integrity and fluid leakage into the interstitium and adjacent tissues [32,33]. Since HS proteoglycans are required for cell growth [34], degradation of HS chains may also hinder mucosal healing following active inflammation [9]. Heparanase is also believed to exert a direct pro-inflammatory effect on macrophages, prompting them to release TNF- α and IFN- γ [30]. Inversely, secreted TNF- α , IFN- γ and IL-1 β stimulate the secretion of heparanase by colonic epithelial cells and the underlying microvascular endothelial cells [30,35].

1.1.2 Current management strategies

Pharmacological and surgical interventions are currently the two main approaches for managing UC [2]. Surgery is usually indicated when patients are unresponsive to medication

and present intractable symptoms or life-threatening complications such as perforation, refractory rectal bleeding, toxic megacolon, fulminant colitis, multifocal dysplasia or cancer [17]. Drugs such as corticosteroids, aminosalicylates and immunosuppressants aim to decrease the level of inflammation [2]. These agents are not always effective in ensuring long term remission and are associated with significant side effects [2,36]. Currently, TNF- α inhibitors (infliximab, adalimumab and golimumab) have been approved by the US Food and Drug Administration for the treatment of steroid-resistant UC or cases where immunomodulatory therapy is ineffective [37]. However, TNF inhibitors are relatively expensive. For example, the cost for infliximab is about US \$1900 for 100 mg. This means that an average adult patient with UC would need to pay US \$6650 for a single dose of infliximab (body weight = 70 kg, 5 mg/kg per dose) [38]. In addition, prolonged use of infliximab is associated with a mildly increased risk of infections and lymphoma [39]. Importantly, approximately 30 to 40% of patients do not respond to infliximab treatment and in up to 40% of patients the initial therapeutic response of infliximab is lost after one to two years [36,39]. Apart from this, approximately 17% of patients treated with infliximab will eventually require a surgical intervention [39].

Influencing migration of lymphocytes using anti-adhesion molecules is an appealing new treatment option for UC [40]. Vedolizumab is a monoclonal antibody that inhibits lymphocyte migration by disabling the function of a transmembrane protein involved in cell adhesion called integrin $\alpha_4\beta_7$. Randomised controlled trials have demonstrated the effectiveness of vedolizumab in both the induction and maintenance of clinical response and remission in active UC without increasing the risk of infections [41]. On the other hand, monoclonal antibodies that target other lymphocyte surface molecules such as CD3 or CD25 have generated only mixed results in patients with UC [36] and evidence of the long-term therapeutic efficacy and clinical safety of these new therapeutic approaches is still lacking.

Overall, there is a need to develop novel treatment strategies for the management of UC which are effective, safe and less expensive than the available therapeutic options. Current treatments are limited in their ability to sustain a remission of symptoms and to prevent recurrent inflammation. The main therapy focus in UC has shifted towards preserving gut function with a reduced risk of hospitalisation, colorectal dysplasia and colectomy [42]. Among the increasing numbers of investigational therapeutic targets, polysaccharides obtained from natural sources are being explored for UC. Initially, the serendipitous observation of improved symptoms in patients with UC treated with unfractionated heparin (UFH) for thrombosis opened up a potential new therapeutic avenue for the treatment of UC [43]. Also, other heparin-like molecules such as fucoidans with notable immune-modulatory and anti-inflammatory activities, have gained considerable interest as alternative therapeutic approach for UC.

1.2 Heparins

UFH has been clinically used as a potent anticoagulant agent for more than 70 years [44]. It belongs to the family of glycosaminoglycans (GAGs), which are a large group of long unbranched carbohydrate polymers [45]. Endogenous heparin is found in the secretory granules of mast cells. It is believed that the heparin regulates the storage and release of mast cell components during tissue injury as a defence mechanism against invading pathogens, rather than for anticoagulation purposes [46]. Commercially available UFH is extracted and purified mainly from porcine intestinal mucosa [47]. Due to the nature, origin and preparation process, UFH is a heterogeneous mixture of unidentified anionic oligosaccharides ranging from a molecular weight (MW) of 3 - 30 kDa, with an average weight of 15 kDa [48]. UFH consists of repeating disaccharide units, N-acetyl glucosamine with either iduronic (90%) or glucuronic acid (10%), and different degrees and patterns of

sulphation (Fig. 1.2A) [45]. Despite its widespread clinical use, UFH is associated with a number of adverse effects such as bleeding and thrombocytopenia. Furthermore, UFH has a low bioavailability when administered other than intravenously and shows an unpredictable dose response [48]. These limitations led to the development of LMWHs, which are prepared by controlled chemical or enzymatic depolymerisation of UFH [49].

Depending on the depolymerisation process, LMWHs are composed of UFH-derived chains with distinct lengths and a MW between 3600 Da and 6500 Da [50]. Therefore, it is important to point out that each of the currently available LMWHs has to be regarded as an individual chemical entity [49]. Due to structural heterogeneity and polydispersity, complete structural characterisation and sequencing of LMWHs or their parent compound, UFH, is extremely complex [49]. Consequently, UFH and LMWHs are generally characterized and labelled in terms of their anticoagulant effect (a measurement of the binding affinity of the components of these agents to the enzymes in coagulation cascade, as anti-thrombin or anti-factor Xa activity) rather than by their MW [51].

Heparin exerts its anticoagulant activity by activating antithrombin [52]. A unique pentasaccharide sequence (Fig. 1.2B) induces conformational change within the antithrombin structure that accelerates its interaction with thrombin and factor Xa by 1000-fold [52]. The major difference between LMWHs and UFH is their relative inhibitory effect on factor Xa and thrombin. Both LMWHs and UFH can inhibit factor Xa, by binding to antithrombin. To inactivate thrombin, UFH must bind to antithrombin and the enzyme, thereby forming a ternary (heparin-antithrombin-thrombin) complex [48]. UFH chains are composed of more than 18 saccharide units so they can easily form the ternary complex. Unlike UFH, LMWH chains are composed of fewer than 18 saccharide units so they can not

bind simultaneously to both thrombin and antithrombin, and hence only inhibit factor Xa but not thrombin [48].

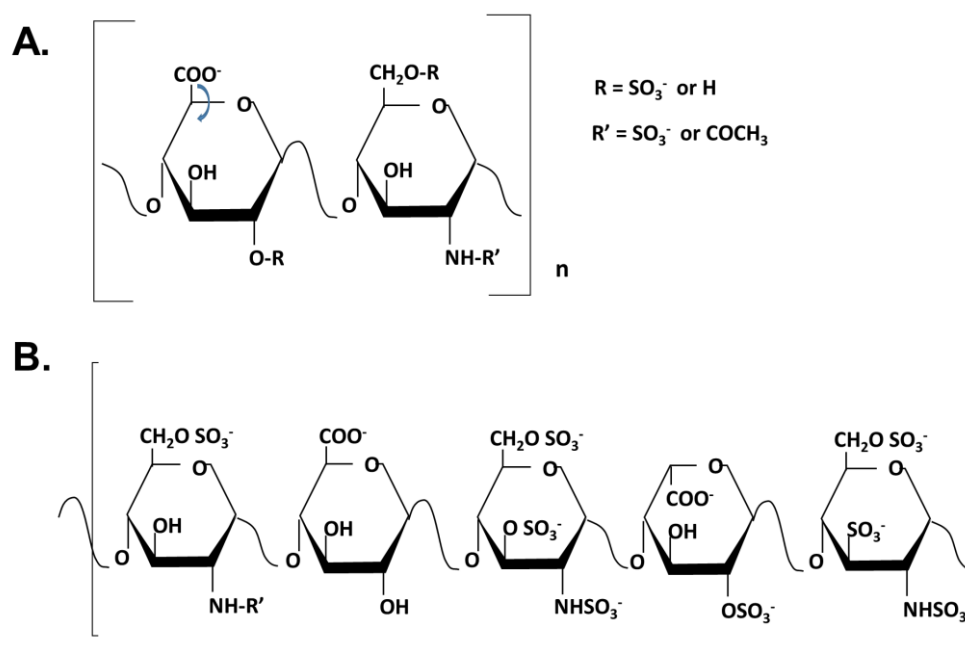


Figure 1.2 Structure of heparins.

(A) The repeating disaccharide unit of heparins: alternating uronic acid (epimerises from glucuronic acid to iduronic acid) and glucosamine, with possible sulphation at R and R' positions. (B) Pentasaccharide anti-thrombin binding sequence of heparins.

1.2.1 Potential mechanism of actions of heparins in UC

The effect of heparins in UC is thought to be based on their anti-inflammatory and mucosal healing activities [53]. Mechanisms by which heparins are postulated to exert their beneficial effects in UC are discussed (Fig. 1.3).

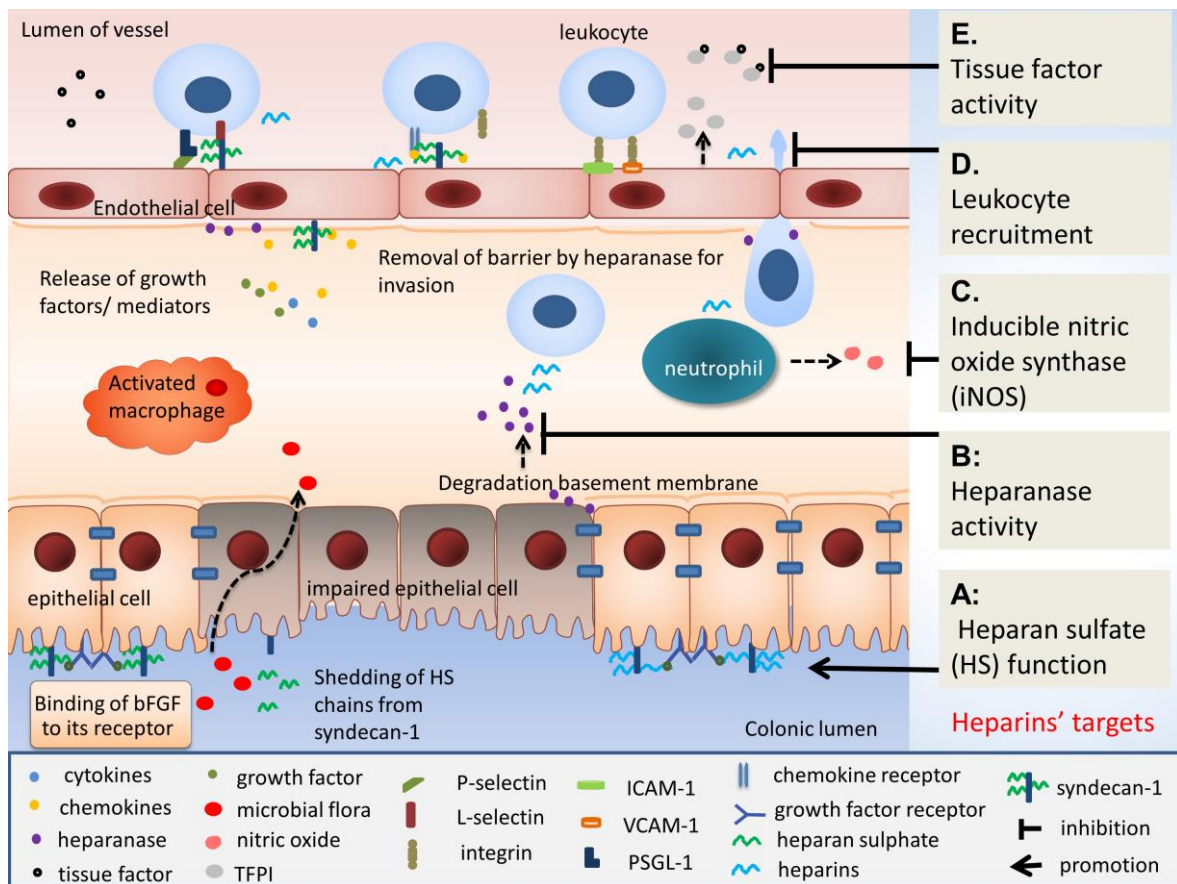


Figure 1.3 Potential heparin-mediated mechanisms in UC.

(A) Syndecan-1 is an important HS proteoglycans present on intestinal epithelial cell surfaces. Syndecan-1 acts as a co-receptor for basic fibroblast growth factor and facilitates the binding of bFGF to its receptor, which stimulate cell growth. In UC, there is shedding of HS chains present on syndecan-1. It is thought that the structural similarity between heparins and HS enables heparins to substitute for lost HS chains, restoring bFGF-FGF receptor binding and assisting mucosal healing in UC. (B) Heparanase, an endo- β -D-glucuronidase capable of cleaving HS chains, is expressed by activated immune cells, epithelial cells and endothelial cells at the site of inflammation. Cleavage of HS chains promotes the release of extracellular matrix-bound growth factors as well as recruitment of immune cells. Since heparins are structurally similar to HS, heparins act as alternative substrates and minimize the degradation of HS chains. (C) Pro-inflammatory cytokines induce nitric oxide production by up-regulating inducible nitric oxide synthase. In UC, NO is released locally by the colonic epithelium, infiltrating neutrophils and macrophages. Heparins reduce the production of NO and protect against inflammation by down-regulating iNOS. (D) During inflammation, leukocytes are recruited from the circulation to the sites of inflammation. Heparins interfere with leukocyte recruitment by interacting with adhesion molecules, as well as inhibiting the release of cytokines and chemokines, thus blocking leukocyte migration into the gut mucosa. Subsequently the influx of leukocytes to sites of inflammation is reduced. Heparins increase the synthesis and release of TFPI.

By blocking the activity of tissue factor, heparins are thought to prevent coagulation as well as inflammation. Basic fibroblast growth factor, bFGF; heparan sulphate, HS; inducible nitric oxide synthase, iNOS; intercellular adhesion molecule-1, ICAM-1; nitric oxide, NO; P-selectin glycoprotein ligand-1, PSGL-1; tissue factor pathway inhibitor, TFPI; vascular cell adhesion molecule-1, VCAM-1.

1.2.1.1 Mucosal healing and barrier function

Mucosal healing has been identified as one of the ultimate therapeutic goals in UC as the inflammation is restricted to mucosa and submucosa [42]. Mucosal healing is essential for the resolution of chronic inflammation in the colon or rectum and thus for the remission of the disease [54]. Mucosal epithelial repair is a complex process involving remodelling of the ECM, angiogenesis, epithelial cell proliferation and differentiation of the colonic mucosa [55]. These processes are initiated by regulatory factors such as cytokines, chemokines and growth factors that interact with HS proteoglycans or HS-rich ECM to restore mucosal integrity and promote epithelial repair [55].

HS proteoglycans play an essential role in sustaining epithelial barrier function and support the basement membrane below [33,56]. Loss of HS proteoglycans from the basolateral surface of intestinal epithelial cells was reported to decrease the integrity of the epithelial barrier, which was associated with the leakage of plasma proteins into the intestinal lumen [33]. However, such leakage was inhibited upon restoring the integrity of HS proteoglycans [33]. As HS proteoglycans expression is significantly altered in UC [57], the integrity of the epithelial barrier is also likely to be compromised. The observed loss of epithelial syndecan-1 during colitis [28,33] is mostly caused by the presence of heparanase and increased levels of pro-inflammatory cytokines, such as TNF- α and IL-1 β [58]. Importantly, reduced levels of HS proteoglycans potentiate the effects of pro-inflammatory cytokines, resulting in a vicious cycle of loss and down-regulation of HS proteoglycans [28].

Among various types of HS proteoglycans, syndecans have a crucial role in wound healing and inflammation [29]. Syndecans consist of a core protein and three covalently linked HS chains. Among syndecans, syndecan-1 is highly expressed on the basolateral surface of epithelial cells and is identified as a co-receptor of basic fibroblast growth factor (bFGF) [59]. This growth factor is a potent stimulator of epithelial cell growth [60,61]. HS chains of syndecan-1 mediate and stabilise the interaction between bFGF and epithelial cell surface receptor for signal transduction of cell growth, as shown in Figure 1.3 [62,63]. However, during active UC, syndecan-1 in the inflamed epithelium is down-regulated predominantly in the reparative epithelium adjacent to ulcer margins [57]. This down-regulation of syndecan-1 reduces the interaction between bFGF and cell surface receptor, resulting in decreased stimulation of cell growth [64].

Interestingly, studies have reported that heparins can substitute for the functions of HS present on syndecan-1 [33,65]. In syndecan-1 knock-out mice with colitis, enoxaparin, a LMWH, improved intestinal wound healing as well as reduced the rate of mortality [65]. Because of the structural similarity, LMWHs are thought to act as functional analogues of HS chains of syndecan-1 [65]. UFH substituted for the lost HS chains that are required for syndecan-1 functions, restoring bFGF-receptor interaction for cell growth *in vitro* [57]. Likewise, subcutaneous enoxaparin significantly decreased the severity of colitis by reducing the colitis-associated shedding of syndecan-1 [66]. These results suggested that heparins could potentially substitute for endogenous HS chains and therefore potentially restore bFGF receptor binding and stimulate epithelial repair in colitis (Fig. 1.3).

1.2.1.2 Inhibition of heparanase activity

It is well-recognised that heparanase activity strongly influences inflammation [67]. Heparanase is responsible for the degradation of HS which is thought to be essential for this

activity [67]. Degradation of HS by heparanase occurs under physiological as well as pathological conditions such as in tissue development and tissue remodelling, wound repair, inflammation and angiogenesis [9]. Normally, heparanase expression is restricted to certain tissues such as activated immune cells and platelets [9]. However, in UC, heparanase is expressed locally at the site of inflammation [31] and also in mice with induced colitis [30]. Overexpression of heparanase in the colon during inflammation degrades ECM, as well as the basement membrane, resulting in barrier damage with ulceration [9]. This temporary disruption of the basement membrane facilitates the migration of leukocytes through the vascular endothelium [29]. Since heparins are structurally similar to HS, they are believed to compete with HS for the binding site of heparanase (Fig. 1.3) [68]. Because of this competition, heparins act as alternative substrate and minimize the degradation of HS [69,70]. Consistent with this model, heparins decrease the degradation of ECM as well as the migration of immune cells to the site of inflammation [68,69].

1.2.1.3 Inhibition of inducible nitric oxide synthase activity

Pro-inflammatory cytokines induces nitric oxide (NO) production by up-regulating inducible nitric oxide synthase (iNOS) [71]. It is reported that iNOS is expressed locally in the colonic mucosa of patients with active UC [72]. Initially iNOS leads to production of NO as a part of the cellular defence against invading antigens [71]. However, under conditions of chronic inflammation, excess levels of NO at the sites of inflammation can cause direct cytotoxic effects. Furthermore, NO reacts with oxygen radicals to form the oxidising agent peroxynitrate which can have damaging effects in large quantities [71]. In this context, heparin-dependent down-regulation of iNOS during inflammation [73,74] was shown to reduce the cellular production of NO and to protect against inflammation (Fig. 1.3) [75,76].

1.2.1.4 Potentiation of tissue factor pathway inhibitor

The link between coagulation and inflammation in UC is still unclear. It has been postulated that chronic inflammation causes imbalance of the coagulation process [77]. Activation of leukocytes during chronic intestinal inflammation leads to propagation and generation of tissue factor [77]. Tissue factor, a transmembrane glycoprotein and cofactor for clotting factor VII/ VIIa, initiates and amplifies blood coagulation, eventually leading to a hypercoagulant state. Beyond its role in coagulation, tissue factor increases the secretion of pro-inflammatory cytokines. These cytokines play an important role in different inflammatory conditions including in colitis [78]. In contrast, tissue factor pathway inhibitor (TFPI), synthesised by endothelial cells, prevents coagulation as well as inflammation. UFH and LMWHs have been shown to increase the synthesis and release of TFPI from endothelial cells (Fig. 1.3) [79]. Therefore, in addition to their anticoagulant role, the release of TFPI could potentially contribute to the observed clinical benefits of heparins against inflammation.

1.2.1.5 Interference with the inflammation cascade: transmigration of leukocytes

The recruitment of circulating leukocytes to sites of inflammation involves a series of sequential events such as active rolling, firm adhesion and migration [29,80]. The translocation of leukocytes (including monocytes, granulocytes and lymphocytes) from the circulation through the endothelium into the inflamed tissue areas is mediated by adhesion molecules activated by pro-inflammatory cytokines [81,82]. Interestingly, heparins are shown to interfere with leukocyte-endothelial cell adhesion and subsequently minimize the migration of leukocytes (Fig. 1.3) [46,53,83].

Active rolling of leukocytes on the inflamed endothelial cell wall involves different lectins, such as L-selectin, P-selectin and E-selectin [29]. L-selectin is constitutively expressed on leukocytes while E-selectin and P-selectin are up-regulated on endothelial cells during inflammation [29]. Selectins interact with cell surface counter ligands of lectins, such as sialyl-Lewis^x, which are tetrasaccharide compounds [83] and HS chains of syndecans [29]. During the rolling process, syndecan expression on the endothelial surface is up-regulated [29]. The HS chains of syndecans, the predominant ligands for L-selectin, mediate the initial contact of circulating leukocytes with the vascular endothelial cells (Fig. 1.3) [29]. Subsequently, chemokines are required to trigger the activation of integrins that cause leukocyte arrest by firm adhesion through the binding of integrins to their ligands on the endothelium [81]. Chemokines, synthesized by activated endothelial cells, immune cells and inflamed tissues during inflammation [84], are bound to HS proteoglycans on endothelial cell surfaces before they can interact with their receptors on the surface of leukocytes [29,56]. As the chemokines bind to their receptors, integrins are activated to bind to their ligands; this assures firm adhesion and thus transmigration of leukocytes across the endothelium to the site of inflammation [29,85].

UFH and LMWH oligosaccharides have been shown to bind selectins [86]. This binding prevents selectins from binding to their ligands and therefore prevents the rolling of leukocytes on endothelial cells [66,87]. Heparins also competitively inhibit chemokines binding to their receptors, resulting in loss of chemotactic activity (Fig. 1.3) [83,88]. Additionally, heparins can effectively attenuate leukocyte-endothelial interactions by inhibiting ligand binding to integrins, as well as the expression of adhesion molecules such as ICAM-I and VCAM-1 (Fig. 1.3) [87,89,90]. As a result, heparins interfere with leukocyte rolling, chemokine-triggered, integrin-mediated firm adhesion of leukocytes to endothelial

cell surfaces, and subsequently reduce the influx of leukocytes to sites of inflammation [88,90].

The effects of heparins on leukocyte migration could be explained by their inhibitory effects on cytokine production. Heparins are reported to suppress the NF- κ B signalling pathway that is responsible for up-regulating the secretion of pro-inflammatory cytokines and chemokines [91,92]. This inhibition resulted in significantly reduced levels of LPS-induced inflammatory mediators such as IL-1 α , IL-6, IL-8 and TNF- α by leukocytes [91,92]. As such, heparins influence multiple pathways to modulate the infiltration of pro-inflammatory immune cells by inhibiting the release of cytokines and chemokines, as well as modulating the function of adhesion molecules. Overall, it can be assumed that all of those activities are, likely to contribute in a synergistic manner to the anti-inflammatory effects of heparins in UC.

1.2.2 Pre-clinical studies of heparins in UC

The effects of heparins in UC have been investigated in murine models of colitis (Table 1.1). These studies differed in: i) experimental designs (e.g. either prevention or treatment protocol); ii) types and doses of heparins used; iii) duration of treatment (5-19 days) and iv) routes of administration (oral, rectal, subcutaneous or intraperitoneal). In the majority of studies, UFH and LMWHs have shown promising therapeutic effect [66,93-99]. Overall, a significant reduction in the severity of colitis with improved macroscopic and histological scores was observed in the treatment groups compared with the untreated colitis group. A remarkably reduced mucosal ulcerations, lesser distortion of crypt or gland structure in the epithelial lining of colon and also decreased myeloperoxidase activity were observed when mice with colitis were treated with heparins [73,96,100]. The ameliorating effects were related to the ability of heparins to facilitate mucosal healing, decrease the levels pro-

inflammatory cytokines such as IL-1 β , IL-6 and TNF- α as well as inhibit infiltration of leukocytes during the inflammatory process [90,91,93,94].

Pellequer and colleagues investigated the effect of enoxaparin in mice with colitis [95]. Mice were treated with enoxaparin either subcutaneously (3 mg/kg/day), orally (6 or 20 mg/kg/day) or rectally (6 mg/kg/day) for 6 days. They showed that enoxaparin when given orally or rectally, the effect was to be superior to subcutaneous enoxaparin. Unlike subcutaneous route, both rectal and oral route of enoxaparin significantly attenuated all aspects of colitis activities and colonic inflammation. Similarly, using other models of animal colitis, it has been demonstrated that oral and rectal routes of heparins can effectively ameliorate colitis [93,94,101]. The possible reason of this observation could be due to high dose of heparins through these routes. Apart from this, these routes of administration enable the delivery of heparins to the site of inflammation for local effect, avoiding systemic exposure thus decreased risk of bleeding. To our knowledge, currently there is no study of the protective effect of oral enoxaparin prior to or simultaneous with the initiation of DSS. On the other hand, Luo *et al.* (2011) demonstrated the effectiveness of 100 mg/kg/day enoxaparin given for 10 days via oral gavage on third day after the onset of acetic acid-induced colitis [94].

Table 1.1 Use of heparins in murine experimental colitis.

Experimental design	UFH/ LMWH (type/dose)	Results	Author and year
DSS-induced colitis in male BALB/c mice	s.c 3.2 mg/kg/day of dalteparin for 5 days	Reduced severity of colitis	Wan <i>et al.</i> 2002 [96]
TNBS- and oxazolone-induced colitis in male BALB/c mice	s.c 3; rectal 6; p.o gavage 6 and 20 mg/kg/day of enoxaparin for 6 days	Reduced severity of colitis in oral and rectal groups	Pellequer <i>et al.</i> 2007 [95]
DSS-induced colitis in male syndecan-1 ^{-/-} and C57BL/6 mice	i.p 5 mg/kg/day of enoxaparin for 8 days	Reduced severity of colitis in syndecan-1 ^{-/-} mice	Floer <i>et al.</i> 2010 [65]
Acetic acid-induced colitis in male and female Kunming species mice	rectal 50 mg/kg/day of a LMWH (124.7 IU/ mg) for 10 days	Reduced severity of colitis	Luo <i>et al.</i> 2010 [93]
Acetic acid-induced colitis in in male and female Kunming species mice	p.o gavage 100 mg/kg/day of UFH (not specified IU/mg), enoxaparin (not specified IU/mg), dalteparin (124.7 IU/ mg) for 10 days	Reduced severity of colitis	Luo <i>et al.</i> 2011 [94]
DSS-induced colitis in male BALB/c mice	s.c 250 µg/kg/day (5 µg/mouse/day) of enoxaparin for 7 days	Reduced severity of colitis	Wang <i>et al.</i> 2011 [66]
DSS-induced colitis in male C57BL/6 mice	s.c 280 µg/kg/day (5 µg/mouse/day) of enoxaparin for 19 days	Reduced severity of colitis	Wang <i>et al.</i> 2013[97]
DNBS- and iodoacetamide-induced colitis in male Wistar rats	s.c 40, 80 or 200 µg/kg/day of enoxaparin, or 100, 200 or 400 IU/kg/day of UFH (not specified IU/ mg) for 7 days	Reduced severity of colitis in 80 µg/kg/day of enoxaparin and 200 IU/kg/day UFH groups	Dotan <i>et al.</i> 2002 [73]
Iodoacetamide-induced colitis in male Wistar rats	s.c 50 - 500 units/kg/day of UFH (not specified IU/ mg) for 7 days	Reduced severity of colitis	Levine <i>et al.</i> 2002 [100]
TNBS-induced colitis in female Sprague-Dawley rats	s.c 0.96 and 1.92 mg/kg/day (150 and 300 IU/kg/day) of dalteparin for 6 days then half the doses the next 7 days	Reduced severity of colitis	Xia <i>et al.</i> 2004 [102]

DNBS-induced colitis in male Wistar rats	Intracolonic parnaparin 0.02-4.5 mg/kg/day (0.005 - 0.9 mg/rat/day), LMWH A, B, C 1.2-3.0 mg/kg/day (0.3 - 0.6 mg/rat/day), and UFH 2.4 - 3.0 mg/kg/day (0.6 mg/rat/day for) 3 days	Reduced severity of colitis	Celasco <i>et al.</i> 2008 [101]
TNBS-induced colitis in male Wistar rats	s.c 4 or 8 mg/kg/day of UFH over 7 days	Reduced severity of colitis	Belmiro <i>et al.</i> 2009 [98]
Acetic acid-induced colitis in male Wistar rats	s.c 4.44 mg /kg/day (500 IU/kg/day) nadroparin (112.5 IU/mg) for 7 and 10 days	Reduced severity of colitis	Yalniz <i>et al.</i> 2012 [99]

Dextran sulphate sodium, DSS; dinitrobenzenesulfonic acid, DNBS; trinitrobenzene sulfonic acid, TNBS; subcutaneous, s.c; intraperitoneal, i.p; oral, p.o.

* dalteparin (156.25 anti-Xa IU/ mg; Fragmin) and enoxaparin (100 IU anti-Xa IU/ mg; Clexane/Levonox) were used in studies unless otherwise specified

1.2.3 Clinical use of heparins in UC

A paradoxical response to UFH in patients with active UC observed more than 30 years ago [103,104] led to investigations of its potential role in the management of this chronic inflammatory condition (Table 1.2). In a 1995 report, Gaffney *et al.* showed improvement in 9 of 10 patients treated with intravenous UFH with remission maintained by subcutaneous administration of UFH [43]. Similarly, Brazier *et al.* reported complete remission in 4 of 6 patients with UC who were treated with UFH [105]. In another study, 13 patients with UC or CD were treated with UFH for a total of 8 weeks [106]. Seven patients with UC, but none of the CD patients, achieved complete remission. One patient with UC required blood transfusion due to the occurrence of heparin-induced major haemorrhage. Evans and co-workers treated 16 cases of refractory colitis with intravenous and subcutaneous (25000 - 40000 IU/ day) UFH [107]. They reported that 12 out of 16 patients improved and 4 patients required surgical intervention. However, 2 patients suffered from bleeding or bruising

complications. Because of the potential of bleeding, Ikehata *et al.* used low dose (5000 IU/day) of UFH for treating patients with active UC refractory to steroid therapy [108]. They reported that low dose UFH was safe and useful when used with standard therapy.

So far three randomised clinical studies investigated the efficacy of UFH in patients with UC [109-111]. In one study, a total of 20 patients (17 severe UC and 3 severe Crohn's colitis) were treated with 5-aminosalicylic acid and either with intravenous UFH for 5 days, followed by subcutaneous UFH for 5 weeks or intravenous hydrocortisone for 5 days followed by oral prednisolone for 5 weeks [110]. UFH was found to be as effective as corticosteroids in treating severe colitis. Importantly, UFH was well-tolerated with no reported bleeding complications. In contrast, Panes *et al.* compared the efficacy of intravenous UFH as monotherapy versus methylprednisolone in hospitalised patients with moderate to severe UC [109]. They found that UFH was not effective as a monotherapy where none of the patients in the UFH group ($n = 12$), compared with 69% of patients in the methylprednisolone group ($n = 13$), achieved significant improvement. In addition, 3 patients in the UFH group dropped out because of increased rectal bleeding. Another study by Korzenik *et al.* evaluated the efficacy of subcutaneous UFH in addition to standard therapy for the treatment of moderate to severe colitis [111]. The results indicated that 3 patients experienced a transient increase in rectal bleeding, but were able to continue UFH treatment.

LMWHs have a better safety profile, including reduced risk of bleeding than UFH [48]. Therefore, subsequent studies investigated the safety, tolerability and efficacy of LMWHs in combination with standard therapy. In one study, 12 patients with mild-to-moderate active UC who had either responded poorly to conventional therapy or had experienced relapse were treated with subcutaneous dalteparin for 12 weeks [112]. This

LMWH was found to be well tolerated and no serious adverse effects were reported. Also, 11 of 12 patients improved symptomatically and 50% of patients achieved complete remission. Subsequently, efficacy of other types of LMWHs was also investigated in UC. For example, Vrij and colleagues reported that 80% of patients with severe active UC responded to subcutaneous nadroparin when administered twice daily for 8 weeks [113]. Alternatively, another study suggested that weekly dosing of subcutaneous enoxaparin was also effective in treating moderately active UC in patients [114].

However, some reports have shown disappointing results. For example, a prospective, double-blind, randomised placebo controlled trial investigating the efficacy of subcutaneous enoxaparin reported no clinical benefit over placebo in mild to moderately active UC [115]. With similar disappointing results, in 2007 de Bievre *et al.* presented a placebo controlled trial in which 29 patients with a mild to moderate relapse of UC during standard treatment were randomised to receive either subcutaneous LMWH (reviparin) or placebo for a period of 8 weeks [116]. The study reported no clinical advantage of the LMWH compared with placebo. Also, data from a larger group of patients (n = 100) revealed that subcutaneous administration of another LMWH (tinzaparin) did not significantly improve clinical outcome in patients with mild to moderately active UC [117].

In the above mentioned studies, UFH or LMWHs were administered either intravenously or subcutaneously since heparins are commercially available as parenteral dosage forms. However, some studies also investigated the efficacy of orally administered LMWHs in UC. In one study, patients with UC (resistant to standard therapy) were treated with oral LMWH twice daily with sulfasalazine for more than 4 weeks. The authors reported that clinical remission was achieved in 95% of patients (n = 20) with no rectal bleeding [118]. Pastorelli *et al.* used an extended colon-released oral formulation of a LMWH

(parnaparin) in patients with mild to moderately active distal UC and refractory to mesalazine. A total of 70% of patients (n = 10) achieved complete clinical remission [119]. The positive results of this formulation of LMWH were further confirmed in a multi-centred, double-blind, randomised, placebo-controlled trial [120].

Shen and colleagues in their meta-analysis of eight randomised controlled trials compared the efficacy and safety of adjuvant parenteral heparins with standard therapy in patients with UC. Their meta-analysis suggested that heparins have no additive benefit over standard therapy although their use was found to be safe in patients with UC [121]. Nonetheless, a Cochrane review by Chande and colleagues concluded that heparins may be effective in UC only if administered as oral colon-release formulation [122].

Table 1.2 Use of heparins in treating patients with UC.

Study design	Patients (n)	UFH/ LMWH (route/type/dosage)	Concurrent treatment with heparins for UC	Effects/ Remission rate	Author and year
Pilot study	Refractory UC (10)	i.v UFH 30000 - 36000 IU daily or s.c 10000 IU bd until advised to stop	Corticosteroids (tapering and stopped), sulfasalazine	Improved clinical outcome (9/10)	Gaffney <i>et al.</i> 1995 [43]
Open label trial	Moderate and severe UC (6)	i.v UFH 3000 IU every 4 hours 1 week then s.c calcium heparinate 0.1 ml/ 10 kg bd 3 weeks or 4 weeks	-	Improved clinical outcome (4/6)	Brazier <i>et al.</i> 1995 ⁺ [105]
Case study	Active UC (16)	i.v or s.c UFH 25000 - 40000 IU daily IU 4 weeks	Corticosteroids or 5- aminosalicylic acids or azathioprine	Improved clinical outcome (12/14)	Evans <i>et al.</i> 1997 [107]
Uncontrolled trial	Mild to moderate UC (12)	s.c dalteparin 5000 anti-Xa IU bd 12 weeks	Corticosteroids or 5- aminosalicylic acids or sulfasalazine	Improved clinical outcome (6/12)	Torkvist <i>et al.</i> 1999 [112]
Uncontrolled trial	UC (13) and Crohn's colitis (4)	i.v heparin, dose aiming a partial thromboplastin time of 60 s for 2 weeks then 12,500 IU bd 6 weeks	Sulfasalazine	Improved clinical outcome (7/13 in UC)	Folwaczny <i>et al.</i> 1999 [106]

Uncontrolled trial	Moderate and severe UC (20)	p.o LMWH 366 IU/ kg bd 4 weeks	Corticosteroid (tapering and stopped) and sulfasalazine	Improved clinical outcome (19/20)	Cui and Jiang 1999 [118]
Open label trial	Moderate active UC (10)	s.c enoxaparin 500 anti-Xa IU/ week for 12 weeks (100 anti-Xa IU/mg)	5-aminosalicylic acids or azathioprine	Improved clinical outcome (10/10)	Dotan <i>et al.</i> 2001 [114]
Open label trial	Severe active UC (25)	s.c nadroparin 5700 anti-Xa IU bd 8 weeks	Corticosteroids, cyclosporins, azathioprine	Improved clinical outcome (20/25)	Vrij <i>et al.</i> 2001 [113]
Open label trial	Active UC (11)	i.v UFH 5000 IU daily 6 weeks	Corticosteroids (tapering and stopped), salicylates and sulfasalazine	Improved clinical outcome (9/11)	Ikehata <i>et al.</i> 2003 ⁺ [108]
Open label trial	Mild to moderate UC (10)	p.o colon-released parnaparin 70,140, 210mg daily 8 weeks (75 - 110 anti-Xa IU/mg)	5-aminosalicylic acids	Improved clinical outcome (8/10)	Pastorelli <i>et al.</i> 2008[119]
Mullti-centre RCT	Moderate and severe UC (70)	s.c UFH 10000 IU bd or tds 6 weeks	Corticosteroids, or 5-aminosalicylic acids or azathioprine	Unknown (result not published)	Korzenik <i>et al</i> 1999 ⁺ [111]
Single-centre RCT	Severe UC (17) and Crohn's colitis (3)	i.v UFH 25000 - 45000 IU daily 5 days then s.c 10000 IU bd 2 weeks then 5000 IU bd 3 weeks	5-aminosalicylic acids	Improved clinical outcome (6/8 UFH treatment group; 8/12 steroid treatment group)	Ang <i>et al.</i> 2000 [110]
Multi-centre RCT	Moderate to severe UC (25)	i.v UFH 31456 ± 290 IU daily 10 days	-	No significant beneficial effect	Panes <i>et al.</i> 2000 [109]

Multi-centre RCT	Mild to moderate UC (41)	s.c dalteparin 5000 anti-Xa IU bd 8 weeks	Corticosteroids and 5-aminosalicylic acids or sulfasalazine	No significant beneficial effect	Torkvist <i>et al.</i> 2001 ⁺ [123]
Multi-centre RCT Double-blind Placebo-controlled	Mild to moderate UC (100)	s.c tinzaparin 175 anti-Xa IU/ kg daily 2 weeks followed by 4500 anti-Xa IU/ day 4 weeks	Salicylates	No significant beneficial effect	Bloom <i>et al.</i> 2004 [117]
Multi-centre RCT Double-blind Placebo-controlled	Active UC (117)	s.c deligoparin 75 or 125 mg daily 6 weeks	Corticosteroids, 5-aminosalicylic acids and/or azathioprine/6-mercaptopurine	No significant beneficial effect	Korzenik <i>et al.</i> 2003 ⁺ [124]
Single-centre RCT	Active UC (34)	s.c enoxaparin 100 anti-Xa IU/ kg daily 12 weeks	Corticosteroids and aminosalicylates	No significant beneficial effect	Zezos <i>et al.</i> 2006 [115]
Multi-centre RCT Double-blind Placebo-controlled	Mild or moderate UC (29)	s.c reviparin 3436 anti-Xa IU bd 8 weeks	Aminoalicylates	No significant beneficial effect	de Bievre <i>et al.</i> 2007 [116]
Multi-centre RCT Double-blind Placebo-controlled	Mild to moderate left-sided active UC (121)	p.o colon-released parnaparin 210 mg (18000-19000 anti-Xa IU) daily 8 weeks	Aminosaliclates	Improved clinical outcome (51/61 in parnaparin treatment group; 38/60 in control group)	Celesco <i>et al.</i> 2010 [120]

Twice daily, bd; three times a day, tds; intravenous, i.v; subcutaneous, s.c; oral, p.o; randomised controlled trial, RCT; ulcerative colitis, UC.

+published as abstract only

1.2.4 Proposed reasons for reported inconsistent clinical outcomes of heparins in UC

The contradictory results observed with heparins are not restricted to UC only; such findings have also been reported in many other types of inflammatory conditions. For example, one randomised clinical study reported an inhibitory effect of inhaled UFH on methacholine-induced bronchoconstrictive response [125]. In contrast, no such inhibitory effect of UFH on bronchoconstriction had been observed in another randomised trial [126]. Also, a LMWH (enoxaparin) had been shown to be an effective and simple alternative therapy for the management of lichen planus [127]. However, there are also reports indicating that heparins are not effective when used for the management of the same clinical condition [128,129].

There could be a number of reasons behind the observed discrepancies with regards to the reported clinical outcomes when heparins were used for the management of UC. These reasons may include the use of: i) different treatment durations (ranging from 10 days to 12 weeks); ii) heparins as monotherapy or combined with standard therapy; iii) different dosage regimens of heparins (e.g. 1 mg/ml of enoxaparin daily for 12 weeks or 5 mg/ml of enoxaparin at weekly intervals for 12 weeks); iv) patient populations with different disease extent and severity (mild, moderate, severe or active or refractory UC) and v) inconsistent study end points (remission of disease symptoms and/or endoscopic and histologic improvement). Apart from the above mentioned well-known reasons, dissimilar outcomes could be because of: i) different routes of administration (subcutaneous, intravenous or oral) and ii) the structural differences between various types of LMWHs.

Heparins are composed of anticoagulant and non-anticoagulant oligosaccharides. The parenteral route of administration is likely to increase the risk of bleeding when an anticoagulant effect was not required. Therefore, low parenteral doses were administered in an attempt to avoid the bleeding complications in patients with UC [108,114]. Also, when

used for the treatment in UC, the availability of heparins at the site of inflammation after parenteral administration is unknown. Alternatively, if heparins are administered orally then they can be delivered to the site of inflammation. This could potentially minimize the risk of bleeding as well as allow the use of high doses. Data from a number of pre-clinical and clinical studies have suggested an increased effectiveness of LMWHs when given orally at high doses [95,119,130]. For example, a specific oral extended-release formulation which avoids degradation and systemic absorption of a LMWH was used to deliver a dose of 210 mg parnaparin (approximately 18000 - 19000 anti-Xa IU activity) to the colonic mucosa of patients with active distal UC [120]. Administration of such a dose would not be possible through parenteral route where the anticoagulant effects predominate. Unlike parenteral heparins, the oral route allows the administration of high dose of LMWHs. This may explain the improved clinical outcome in patients with UC who received oral LMWHs compared with patients who were treated with intravenous UFH or subcutaneous LMWHs.

A total of eight LMWHs are approved for clinical use worldwide, as shown in Table 1.3. All LMWHs are prepared through either chemical or enzymatic depolymerisation and have average MWs between 3800 and 6500 Da [49,131]. They display many similar physical, chemical and biological properties [49,51]. However, many studies have shown structural and compositional differences between various types of LMWHs [49,132]. Apart from this, the type of UFH chosen to be chemically or enzymatically digested and the degree of purification of the starting material both contribute to the final characteristics of the particular LMWH. One type of LMWH (e.g. tinzaparin) is structurally different than another type of LMWH (e.g. dalteparin) and therefore the clinical efficacy of two LMWHs is not comparable. For example, subcutaneous administration of dalteparin twice daily for 12 weeks was found to be effective and safe in patients with active, corticosteroid-refractory UC [112]. On the other hand, tinzaparin showed no benefit over standard therapy when used

for the treatment of active UC [117]. The presence of structurally different oligosaccharides in dalteparin and tinzaparin could be responsible for the observed disparity. For example, dalteparin is prepared by nitrous acid induced deaminative cleavage of UFH resulting in formation of an anhydromannitol ring at the reducing end [133]. Tinzaparin is prepared by enzymatic β -eliminative cleavage by heparanase I of UFH, resulting in the presence of an unsaturated uronate residue at the non-reducing end of virtually every chain [133]. Apart from this, tinzaparin is much more polydisperse in nature than dalteparin and is composed of oligosaccharides ranging from dp2 (two saccharide units) to dp18 (18 saccharide units). On the other hand, dalteparin does not have oligosaccharides smaller than dp8 and is mainly composed of oligosaccharides larger than dp10 [133]. Therefore it may be possible that the clinical effect of tinzaparin observed in patients with UC could be because of the presence of: i) smaller oligosaccharides ($< dp\ 6$) and/ or ii) the unique unsaturated uronate residue at the non-reducing end. Several *in vitro* studies have also shown that the presence of different proportion of large and small oligosaccharide chains within various LMWHs can produce different responses to the cells and molecules involved in the process of inflammation [134,135].

Table 1.3 Different LMWHs with their methods of depolymerisation, average molecular weight and anticoagulant activity [49,51,136,137].

Product	Mode of depolymerisation	Average molecular weight (MW)	European pharmacopeia (EP) range for MW	Anti-Xa IU/mg	Anti-factor Xa/ anti-thrombin	EP range for anti-factor Xa/ anti-thrombin
Tinzaparin	β -eliminative cleavage by heparinase I	6500	5500 - 7500	70 - 120	1.5	1.5 - 2.5
Enoxaparin	β -eliminative cleavage of benzyl ester of UFH by sodium hydroxide (NaOH)	4500	3800 - 5000	90 - 125	3.9	3.3 - 5.3
Dalteparin	Deaminative cleavage with nitrous acid	6000	5600 - 6400	110 - 210	2.5	1.9 - 3.2
Parnaparin	Oxidative depolymerisation with copper (II) ion (Cu^{2+}) and hydrogen peroxide (H_2O_2)	5000	4000 - 6000	75 - 110	2.3	1.5 - 3.0
Nadroparin	Deaminative cleavage with nitrous acid	4300	3600 - 5000	95 - 130	3.3	2.5 - 4.0
Certoparin	Deaminative cleavage with isoamyl nitrite	5400	n.a	95 - 130	2.4	n.a
Reviparin	Deaminative cleavage with nitrous acid	4400	n.a	106 - 130	4.2	n.a
Bemiparin	β -eliminative cleavage of quaternary ammonium salt of UFH by cetyltrimethylammonium ion (CTA^+) and hydroxide (OH^-)	3800	n.a	80 - 120	9.7	n.a
Not available, n.a						

The clinical efficacy of enoxaparin, one of the most widely used LMWHs for the treatment and prophylaxis venous thromboembolism, in the management of UC has been investigated. Dotan *et al.* found that enoxaparin when administered once a week combined with aminosalicylate (mesalamine) was an effective adjuvant treatment for UC [114]. They also concluded that a combination of enoxaparin and mesalamine therapy may delay or preclude the need for steroid treatment. However, Zazos and co-workers observed that daily administration of enoxaparin combined with aminosalicylate had no additional benefit over standard therapy (aminosalicylate and weekly tapered corticosteroids) [115]. The precise reason for the varying response to enoxaparin is currently unknown. However, one possible explanation could be the batch-to-batch variability of enoxaparin preparations. For example, in one study the clinical effect of enoxaparin in chronic inflammation was observed only when it was used from certain batches [138]. A LMWH is composed of a mixture of anticoagulant and non-anticoagulant oligosaccharides [76,134]. It is now known that the presence of non-anticoagulant oligosaccharides is responsible for the reported anti-inflammatory activity of a LMWH [134]. Commercially available LMWHs are standardised according to their anticoagulant oligosaccharides (responsible for the anticoagulant effect) but not based on their non-anticoagulant oligosaccharides [132,139]. Therefore, each batch of enoxaparin is expected to have the same anticoagulant activity but not the same non-anticoagulant effect (e.g. anti-inflammatory activity). In fact, Patel *et al.* have reported significant structural and compositional differences between two different batches of enoxaparin formulated by the same manufacturer [140]. Therefore, the clinical effect of enoxaparin in UC may be dependent on the amount and nature of anti-inflammatory oligosaccharide(s) present in any given batch of enoxaparin, which may account for the varied responses seen.

1.2.5 Complexity of heparins

Apart from the reported inconsistent clinical outcomes, the potential of heparins in UC are largely hindered by the fear of bleeding from the anticoagulant activity. The investigation of anti-inflammatory effects of heparins is challenging because these activities are expressed with high heparin concentrations where the anticoagulant effects predominate. To avoid the bleeding complications, clinical studies have used low doses of UFH or LMWH to assess their roles in the management of UC. Because of the considerable therapeutic potential of high doses of LMWHs in the management of UC, efforts have been made to identify and isolate the non-anticoagulant oligosaccharides of LMWHs. The currently used approach to identify the non-anticoagulant fractions is further depolymerisation of LMWHs by chemical or enzymatic methods. Nonetheless, the depolymerisation process inflicts compositional changes in oligosaccharides of heparins [133] and it has been shown that this process was responsible for alteration of the structures of oligosaccharides important for certain biological activities of intact heparins [141,142]. The depolymerisation is carried out at elevated temperatures. Patel and colleagues found that some oligosaccharides in LMWH are heat sensitive and can undergo chemical modification, especially desulphation [143]. An oligosaccharide's sulphation pattern is a key characteristic feature for its anti-inflammatory properties [134]. Apart from this, depolymerisation follows the freeze-drying process [144]. The freeze-drying results in physical changes of some oligosaccharides within the LMWH molecule.

To separate, isolate and identify the non-anticoagulant oligosaccharides of LMWH without prior chemical or enzymatic digestion is another effective approach. The main obstacle in the isolation of LMWH oligosaccharides is the deficiency of a high resolution technique. LMWHs have proven to be extremely difficult to analyse because of their high

negative charge and structural complexity [145]. These characteristics make the separation of LMWHs without further depolymerisation prior to the analysis, a difficult analytical challenge. A reversed-phase ion-pair high performance liquid chromatography method has been developed and validated for the separation and isolation of various oligosaccharides of LMWHs without prior enzymatic or chemical depolymerisation [146]. However, this technique requires a positively charged non-volatile ion-pairing reagent to separate negatively charged oligosaccharides of LMWH. The presence of non-volatile ion-pairing reagent is reported to interfere with the investigation of anti-inflammatory properties of the isolated oligosaccharides [147].

More recently an ion-exchange chromatography (IC) technique for the separation of a LMWH has been developed [76]. This technique successfully separated enoxaparin into more than 20 different oligosaccharides. Using the same technique, another study has also identified groups of oligosaccharides in LMWHs with high, low and no anticoagulant activities [134]. Each non-anticoagulant oligosaccharide should then be examined for its clinical efficacy against UC. Such an investigation may lead to new formulations of a purified LMWH oligosaccharide (or combination of purified oligosaccharides) that exhibit effective anti-inflammatory properties, without significant anticoagulant activity. The development of such a formulation could therefore serve as a novel therapeutic approach for the treatment of UC, as well as other inflammatory diseases.

In addition, when a commercially available LMWH is tested for its efficacy against UC it is important to have the presence of the same non-anticoagulant oligosaccharides in different batches. An effective way seems to be to obtain the standard chromatographic profiles of non-anticoagulant oligosaccharides of the particular batch of a LMWH (e.g. enoxaparin) with a proven value in UC. To minimize batch-to-batch variations of non-

anticoagulant oligosaccharides and hence inconsistent clinical outcomes, enoxaparin batch-to-batch uniformity could be confirmed by comparing the chromatographic profiles of enoxaparin's non-anticoagulant fragments against its non-anticoagulant oligosaccharide(s) with proved anti-inflammatory effect.

1.3 Fucoidans

Like heparins, fucoidans are heterogenous and highly sulphated polysaccharides. Unlike heparins, fucoidans are naturally present in the cell wall matrix of brown algae [148]. Fucoidans are synthesised in the Golgi apparatus of cells as a kind of mucilage. It is believed that the main function of fucoidans is to prevent the plant from drying out when exposed to air. Although fucoidans are also found in the cell wall of invertebrates such as sea cucumber and sea urchin, there is only very limited information about their function in these organisms [148]. Fucoidans have been extracted from different brown algae such as *Fucus vesiculosus*, *Fucus evanescens*, *Cladosiphon okamuranus*, *Undaria pinnatifida*, *Ecklonia cava* and *Laminaria japonica*. Commonly, seaweeds are collected, cleaned, and dried before fucoidans are extracted using water or organic solvents with or without chemical or enzymatic hydrolysis [149]. Thus, the products derived from brown algae are mostly fucoidan containing extracts rather than fully purified fucoidans.

In contrast to heparins, fucoidans are composed primarily of repeating units of disaccharide units with α -1,3-linked and α -1,4-linked fucose (Fig. 1.4) [148]. Apart from fucose, minor amounts of uronic acids, glucosamines and monosaccharides such as galactose, xylose, glucose and mannose are also present [150,151]. Unlike heparins with probable sulphation at N-, 2-O and 6-O of disaccharide units, sulphation of fucoidans is found at 2-O, 4-O or both positions and in rare cases at the 3-O position [152]. Neither N-acetylated nor N-sulphated groups are present in fucoidans compared with heparins. The

polysaccharide chains of fucoidans are not only linear but also highly branched. The MW of fucoidans differ from one species to the other, ranging from 13 kDa to 1300 kDa [153]. Also, MW within the same species can be different. For example, crude fucoidan from *Fucus vesiculosus* purchased from Sigma has MW 20 - 200 kDa, with an average weight of 140 kDa. Others reported that fucoidan prepared from *Fucus vesiculosus* has a MW between 43 kDa and 680 kDa, with average of 160 kDa [154]. The differences might be due to the extraction method used, season of collection or be based on geographical differences. Because of the compositional diversity of crude fucoidans and their large size, attempts have been made to prepare low molecular weight fucoidans (LMWFs) to facilitate the identification of the structural basis of their biological activities. Similar to LMWHs, LMWFs can be obtained by chemical, physical or enzymatic depolymerisation of intact fucoidans [155].

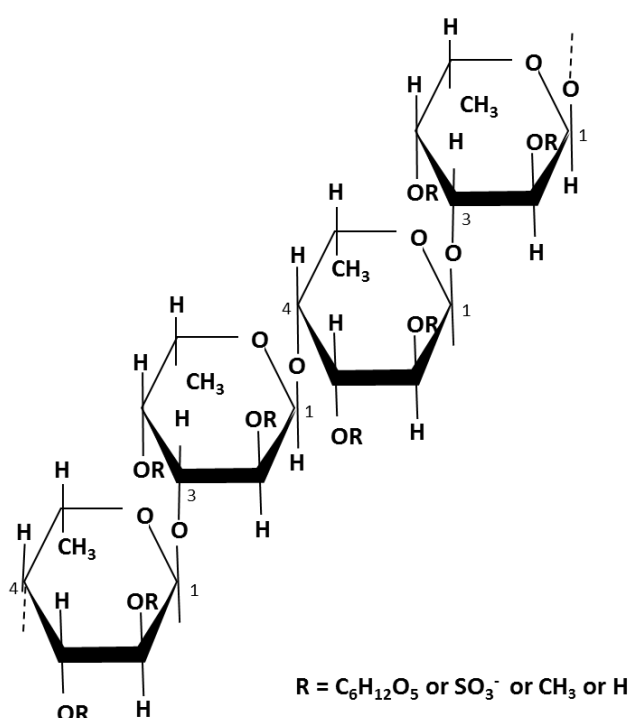


Figure 1.4 Structure of fucoidans.

Typical backbone chain of fucoidans in brown algae consisting of repeating disaccharide unit of α -1,3-fucose or α -1,4-fucose. R depicts the sites for potential substitution of fucose unit, sulphate or acetyl groups [151].

With recent attention regarding the usefulness of marine algae, fucoidan-rich extracts from brown seaweeds such as *Fucus vesiculosus* have become commercially available as dietary products and health supplements. It is believed that fucoidans enhance immune functions by modulating physiological responses [156]. Like heparins, fucoidans possess abundant biological activities ranging from anti-inflammatory, anti-oxidant, anticoagulant, anti-bacterial, anti-viral to anti-cancer properties [151,156]. Recently, a randomised, placebo-controlled, double-blind trial found increased immune responses to seasonal influenza vaccination in elderly volunteers who were supplemented with fucoidan extract from *Undaria pinnatifida* [157]. Their findings demonstrate the immune-stimulatory effect of fucoidan, echoing a possible role of fucoidan in immune-potential. Therefore, fucoidans appear to possess potential as health promoting or functional foods and as ingredients in cosmoceutical products. Recently, scientific interest has shifted towards understanding their value as pharmaceutical drugs [156,158]. At present, researchers have evaluated the potential of fucoidans in various therapeutic applications including inflammation, infection and cancer.

1.3.1 Fucoidans in inflammation

Fucoidans have been reported to reduce inflammation in different animal models of inflammatory conditions. Intravenous injection of 4 mg/kg/day of fucoidan extracts from nine different species of brown algae such as *Fucus evanescens*, *Fucus serratus*, *Fucus vesiculosus*, *Laminaria saccharina* and *Cladosiphon okamuranus* were compared in an acute model of peritonitis in rats. Each fucoidan extract reduced the number of neutrophils recruited into the cavity to different extent (66.4% - 94.1% inhibition) [151]. On the other hand, intravenously injected fucoidan from *Fucus vesiculosus* at 25 mg/kg prior and immediately after pancreatitis induction reduced the severity of inflammation in mice by

decreasing neutrophil infiltration and systemic inflammation [159]. Similarly, orally administered 300 mg/kg/day of fucoidans extracted from *Undaria pinnatifida* inhibited inflammation by suppressing the production of inflammatory cytokines in an arthritis model in mice [160]. Moreover, in a zebrafish model of tail-cutting acute injury and LPS-induced inflammation, fucoidan extract from *Ecklonia cava* and *Fucus vesiculosus* at 100 µg/ml respectively inhibited inflammation significantly [161]. Overall, the anti-inflammatory properties of fucoidan extracts appear to be similar to those of heparins.

Likewise, the mechanisms by which fucoidans attenuate inflammatory responses are thought to be similar to the action of heparins. In parallel with heparins, fucoidans extracted from different sources such as *Fucus vesiculosus* and *Laminaria japonica* down-regulate the NF-κB signalling pathway and hence reduce the levels of a number of pro-inflammatory cytokines such as IL-6 and TNF-α and matrix metalloproteinases (MMPs) [162-164]. Fucoidans interfere with P- and L-selectins, thus prevent leukocyte rolling on the endothelium before their adhesion [165] and extravasation from circulation into the inflamed site [159,166]. The anti-inflammatory effect of fucoidans is also attributed to the suppression of iNOS expression by immune cells, which inhibits their NO production [165]. Nonetheless, the cellular and molecular mechanisms of fucoidans and in particular their target molecules that are responsible for the different biological activities remain poorly understood.

1.3.2 Fucoidans in UC

In contrast to heparins, the use of fucoidans in UC has not yet been investigated in patients with UC. Given that oral fucoidans were found to be safe in pre-clinical and other clinical studies as well as the success of fucoidans in immunomodulation and improvement of osteoarthritis [156], it is likely that fucoidans could be used to treat chronic inflammatory conditions such as UC.

Several studies have investigated the effects of fucoidans in animal models of UC. Fucoidan extracts from a number of brown algae were shown to prevent or treat colitis (Table 1.4). Not surprisingly, their specific activity was dependent on the source of the extracts. Administering chow containing 0.05% w/w fucoidan obtained from *Cladosiphon okamuranus* was reported to be more effective than fucoidan derived from *Fucus vesiculosus* in relieving the pathology of chronic colitis in mice [167]. It is generally thought that fucoidan ameliorates intestinal inflammation by down-regulating the production of pro-inflammatory cytokines such as IL-6 by colonic epithelial cells. In addition, it was also demonstrated that intravenous *Fucus vesiculosus* extract at 25 mg/kg/day reduced mucosal inflammation in acute colitis by abolishing TNF- α -induced rolling and migration of leukocytes [166].

Oral fucoidan extract from *Laminaria japonica* (300 mg/kg/day) was reported to reduce the overall inflammatory scores in an acute colitis mouse model, which was associated with decreased levels of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α , however this effect was not seen at a lower dose (100 mg/kg/day) in this model [168]. In contrast, oral administration of a fucoidan extract of an undisclosed source at 20 mg/kg/day was found to be effective in ameliorating colitis in the same mouse model. Interestingly, a greater protective effect against colitis in mice was also observed when fucoidan extract was given in combination with lutein, a xanthophyll and one of known naturally occurring carotenoids present in fucoidan extracts, which suggests that extracts from algae might be composed of substances having synergistic anti-inflammatory effect [169].

Table 1.4 Fucoidans in murine experimental colitis.

Experimental design	Fucoidan (type/dose)	Results	Author and year
DSS-induced acute colitis in male Balb/c mice	i.v 25 mg/kg/day <i>Fucus vesiculosus</i> fucoidan (Sigma) prior 5 days and during 5 days colitis induction	Reduced severity of colitis	Zhang <i>et al.</i> 2001 [166]
DSS-induced chronic colitis in female Balb/c mice	p.o via chow containing of 0.05% w/w <i>Cladosiphon okamuranus</i> fucoidan (Tropical Technology Centre) or <i>Fucus vesiculosus</i> fucoidan (Sigma) for 56 days	Reduced severity of colitis with <i>Cladosiphon okamuranus</i> fucoidan	Matsumoto <i>et al.</i> 2004 [167]
DSS-induced acute colitis in male Balb/c mice	p.o 20 mg/kg/day fucoidan (species unspecified, Haewon Biotech) alone or in combination with Lutein (Yixin Pharmaceutical) for 4 days after colitis induction (day 8 to day 11)	Reduced severity of colitis	Lee and Yoon 2011 [169]
DSS-induced acute colitis in male Balb/c mice	p.o 100 or 300 mg/kg <i>Laminaria japonica</i> fucoidan (Kyung Hee Hanyak Company) alone or in combination with probiotic mixture containing 3 bacterial genera (<i>Bifidobacterium</i> , <i>Lactobacillus</i> and <i>Streptococcus</i>) twice daily for 7 days during colitis induction	Reduced severity of colitis in 300 mg/kg alone or in combination groups	Ko <i>et al.</i> 2014 [168]

Dextran sulphate sodium, DSS; intravenous, i.v; oral, p.o.

1.3.3 Prospects and challenges in the development of fucoidan-based therapy in UC

Unlike heparin, fucoidans are mainly available as oral formulation at present. Based on the experiences with heparins, it is assumed that the absorption and bioavailability of fucoidan is poor due to their high anionic polarity and large molecular size. However, low bioavailability is not an issue in UC where local effect is preferable over systemic exposure to a drug. Nonetheless, free fucoidan has been detected in human plasma after consumption of whole *Undaria pinnatifida* extract (3 g/ day) by healthy volunteers [170]. It was proposed that gut bacteria degrade fucoidan chains into smaller fragments, which are then absorbed

into the blood stream. However, the antibody used for detection of fucoidans could have cross-interacted with endogenous heparins. Without compositional analysis, the pharmacokinetic profile of each fucoidan, its degradation products and metabolites after oral administration are not known. Importantly, based on their similarity to heparins, oral administration of fucoidan is not associated with the risk of bleeding. A pilot study in healthy humans reported that fucoidan, when given orally, did not demonstrate anticoagulant activity [171]. Nevertheless, it was shown that when tested *in vitro*, fucoidan extracts from some species exhibit heparin-like anticoagulant activity by a mechanism that is not completely understood.

Since fucoidans share some structural similarities and anionic characteristics with heparins, it was postulated that the position of sulphate groups, degree of sulphation and MW of fucoidans are essential for their interaction with proteins, thereby, eliciting complex biological responses [148]. Indeed, the biological properties of fucoidans are dependent on the chemical compositions of different fucoidans [172]. In a comparison study of fucoidan extracts from nine different origins, a distinctive specificity and selectivity of each extract with regards to anti-inflammatory, anti-angiogenesis, anti-coagulant and anti-adhesion was reported [151]. The extracts from *Laminaria saccharina* and *Fucus serratus* effectively exhibited inhibitory activities in all assays. However, while the extract of *Fucus vesiculosus* strongly inhibited neutrophil infiltration in rats, they displayed no other activities *in vitro*. While fucoidan extracted from *Cladosiphon okamuranus* demonstrated a potent anti-inflammatory activity, it was less effective as an anti-adherent and had no anti-angiogenesis and no anti-coagulation effects. It was speculated that the loss or lack of activities was related to reduced sulphate content and the presence of glucuronyl substituents along the polysaccharide chains of *Cladosiphon okamuranus* [151]. When the anti-inflammatory activity of fucoidan extracts from three seaweeds were compared for their effects on NO

production in LPS-induced RAW264.7 macrophages, an extract from *Sargassum cristaefolium* showed strong inhibitory effects, while the others had low or no activity. The optimum sulphate content was estimated to be 9.42%, while higher sulphate content or oversulphated fucoidan extracts had less NO inhibitory activity [173]. In term of size, fucoidans with a MW between 380 kDa and 1200 kDa showed higher NO inhibitory activity than the LMWHs between 1.9 and 106 kDa. Others also demonstrated that higher MW fucoidan (> 150 kDa) exhibited stronger cytotoxicity against cancer cells compared with a LMWH (27 - 81 kDa) derived from *Undaria pinnatifida* [174]. In contrast, in a study comparing the activity of different fucoidan fractions from *Undaria pinnatifida*, lower MW fractions (5 - 30 KDa) were considerably more active in inhibiting cell growth compared with fractions with higher MW (> 30 KDa) [175]. Interestingly, different MW fucoidans extracted from *Undaria pinnatifida* showed distinctive activities in an arthritis model in mice [160]. High MW fucoidan (100 ± 4 kDa) increased inflammation by enhancing the production of IFN- γ in spleen cells, whereas the LMWF (1 ± 0.2 kDa) had the opposite effect.

The variation of responses might be attributed to the differences in chemical features and compositions present in different crude fucoidan preparations. Fucoidans are present in more than a thousand different species of brown seaweeds [155]. Apart from the sources, location, season they are harvested, the extraction and purification process add another dimension to the complexity of their polymeric structure, composition as well as their purity. Since fucoidans are extracted differently in the literature, each isolated crude fucoidan preparation described by different researchers is unique in their chemical composition and structure. In addition, seaweeds are known for having other bioactive substances like lutein and polyphenols apart from fucoidan, thus numerous additional components could be included in fucoidan extracts, which could be confounding factors for the observed effects

of fucoidans. Thus it is important to note that the sourcing process is the key factor that influences the compositions (sequence, sulphation pattern and MW) of fucoidan extracts, and ultimately their biological activities.

It has become clear that the structural elucidation of fucoidans will be essential for a thorough understanding of their biological properties. The availability of analytical techniques such as mass spectrometry, infrared spectroscopy and nuclear magnetic resonance spectroscopy has facilitated the structural analysis of fucoidans [158]. These powerful tools provide structural information including the composition of their backbone monosaccharides, glycosidic linkage types, positions of sulphation and branching. However, studies reveal that the chemical compositions and structures of fucoidans are very complex, vary from species to species and highly dependent on the sources and preparation processes [158,176]. Like in the case of heparins, to facilitate structural and compositional analysis, both chemical and enzymatic methods are used to partially hydrolyse fucoidans into smaller fragments and to remove sulphate groups [176]. However, this is not a straightforward process as depolymerisation may introduce changes to the sulphation pattern, to oligosaccharides sizes and their residues. Therefore, similar to the situation with heparins, due to the complexity of this polymeric mixture, the precise structural characterization of fucoidans will be challenging. In fact, given the branched nature of fucoidans, the structural activity relationships will be significantly more difficult compared with heparins.

1.4 Rationale of study

UC involves dynamic process of inflammation and requires continuous optimisation of treatment throughout the course of disease. Therapeutic agents with different mechanisms of action would be important to maintain long-term remission. Heparins and fucoidans possess anti-inflammatory activities and are therefore potential therapeutic candidates for

UC. However, at present, the uncertainty around the effectiveness of heparins and fucoidans in UC make them unsuitable for the management of UC. This is further complicated by their heterogeneity. It is estimated that only 30% of oligosaccharides present in heparins actually possess anti-coagulation activity, and the remaining non-anticoagulant oligosaccharides may have other physiological functions such as anti-inflammatory activity. Commercially available UFH and LMWHs are standardised according to their anticoagulant activity but not tested for the non-anticoagulant properties. Each batch of UFH or a LMWH is expected to have a similar composition with regards to their anticoagulant oligosaccharides. However, this is not the case when the composition of non-anticoagulant oligosaccharides is considered. Furthermore, heparins may lead to bleeding when used in clinical conditions such as UC where the anticoagulation is not required. Therefore, in order to minimise the risk of bleeding and improve the specificity of heparins in UC, it is important to identify the oligosaccharides of heparins that possess anti-inflammatory activity without the anticoagulant side effects. Similarly, fucoidans, extracted from seaweed, are crude mixtures of oligosaccharides. Their composition is highly dependent on the species, site and season of collection as well as extraction and purification methods. Likewise, because of their heterogeneity, fucoidans remain underexploited as an alternative therapeutic. However, commercially available fucoidan extracts are claimed to improve gut health and could be potentially useful for patients suffering from intestinal inflammation. Thus, it is important to investigate and confirm their potential in UC. These necessitate pre-clinical studies of heparins and fucoidans as a first step towards introducing these compounds as novel therapeutic approaches for chronic inflammatory disorders.

1.5 Aim and objectives

An ideal therapeutic agent for UC should be: i) effectively relieve acute inflammation, induce and maintain clinical remission; ii) improve mucosal healing while prevent the long-term complication of colorectal cancer; iii) safe or free from unwanted adverse events; iv) available as an oral formulation which improves patient compliances and avoids the inconvenience of parenteral injections; v) stable and allow targeted delivery to the site of inflammation and vi) economical and easily accessible.

Therefore, the overall aim of this project was to investigate the efficacy of sulphated polysaccharides (enoxaparin and fucoidans) in a model of experimental colitis with the ultimate goal of identifying specific oligosaccharides of enoxaparin that are responsible for any anti-colitis activity observed.

To achieve this, we:

- a) examined and compared the effects of UFH, LMWHs (enoxaparin and dalteparin) and structurally unmodified oligosaccharides of enoxaparin on human colonic epithelial cells *in vitro*.
- b) compared the effectiveness of enoxaparin given either intraperitoneally or orally via the diet in colitis.
- c) evaluated the effect of fucoidan extracts given intraperitoneally or orally in colitis.
- d) tested the effect of different oligosaccharides of enoxaparin in colitis.

Chapter 2

Chapter 2 Identification of Pro- and Anti-proliferative Oligosaccharides of Heparins

2 Abstract

Heparins, unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) are heterogeneous mixtures of anticoagulant and non-anticoagulant oligosaccharides. In addition to their well-known anticoagulant effect, heparins have shown to mediate a wide range of non-anticoagulant effects, including the modulation of cellular growth. However, contradictory results have been reported with regards to their effects on cellular proliferation, with some studies suggesting anti-proliferative, while others indicating pro-proliferative effects. This study investigated the proliferation of human colonic epithelial cancer cells in the presence of UFH and LMWHs (enoxaparin and dalteparin). In our experimental setting, all heparins caused a dose-dependent reduction in cellular growth, which correlated well with the induction of cell cycle arrest in G₁ phase and which was not associated with significant changes in cell viability. The effects on cellular proliferation of 14 different oligosaccharides of enoxaparin obtained through ion-exchange chromatography were also assessed. Surprisingly, only two oligosaccharides showed distinctive anti-proliferative effects while the majority of oligosaccharides actually stimulated proliferation. Interestingly, the smallest oligosaccharide devoid of any anticoagulant activity showed the strongest anti-proliferative effect. Notably, heparins are currently standardised only according to their anticoagulant activity but not based on other non-anticoagulant properties. Our results indicate that slight differences in the composition of heparins' non-anticoagulant oligosaccharides, due to different origins of material and preparation methods, have the potential to cause diverse effects and highlight the need for additional characterisation of non-anticoagulant activities.

2.1 Introduction

Unfractionated heparin (UFH) and low-molecular-weight heparins (LMWHs) have been the subject of considerable research interest with regard to their structural and biological properties [46]. UFH has been clinically used as an anticoagulant for more than 65 years. It belongs to the family of GAGs and it is a heterogeneous mixture of structurally unidentified anionic oligosaccharides [45]. UFH with an average mass of 15 to 20 kDa is extracted from animal tissues including bovine lung and porcine intestinal mucosa [49]. Despite the widespread use of UFH, it is associated with a number of problems including drug-induced adverse effects, low bioavailability and unpredictable dose response [48]. These problems have led to the development of LMWHs, which are obtained by controlled enzymatic or chemical depolymerisation of UFH [49]. LMWHs have an average MW ranging from 3600 to 6500 Da [44]. In the current clinical settings, LMWHs have replaced UFH to a large extent because of their favourable pharmacokinetic properties and fewer adverse effects [48]. All LMWHs are distinct from each other depending on the tissue source of the parent UFH, and the methods and conditions employed for their preparation [49].

It has now been well recognised that in addition to their well-known anticoagulant activity, heparins (UFH and LMWHs) possess a wide range of biological properties, including their ability to modulate the cellular growth [46,177]. Heparins have mostly shown inhibitory effects on the proliferative capacity of cells originating from different tissues and organisms [178,179]. For example, multiple studies have demonstrated that heparins inhibited the proliferation of smooth muscle cells derived from bovine, porcine, rat and human tissues [142,178,180-182], human normal osteoblast cells [183,184], human bone marrow derived mesenchymal stromal cells [185] and human mesenchymal stem cells derived from bone [186]. The mitogenic activity of rodent, bovine and human endothelial

cells was significantly inhibited in the presence of heparins [178,179,187]. Although the dose response varied depending on the specific cell types used, most studies generally agreed that the inhibition of cell proliferation by heparins is concentration-dependent [178,181]. Interestingly, this anti-proliferative property of heparins has also been observed in cancer cells, which has sparked interest in a potential therapeutic use of heparins for treating human malignancies [177]. Multiple studies reported that UFH and LMWHs, such as dalteparin, enoxaparin and nadroparin, decrease the proliferation of human lung adenocarcinoma cells [188-190], murine mammary adenocarcinoma cells [191], human malignant mesothelioma cells [192], human osteosarcoma cells [184] and primary human glioma-derived cells [193].

Despite a number of studies reporting that heparins exert anti-proliferative effects, surprisingly, some studies have indicated that heparins can also possess the opposite effect. For example, it was reported that UFH can stimulate human intestinal cancer cell growth [194,195] and can increase the proliferation of primary rat intestinal cultures in a dose-dependent manner [196]. In addition, some studies have reported no change in cell proliferation in the presence of heparins [197-199]. Therefore, the current literature on the regulation of cell growth by heparins remains contradictory at present.

Heparins are heterogeneous mixtures of sulphated oligosaccharides. Along with the oligosaccharides possessing anticoagulant properties, a large number of non-anticoagulant oligosaccharides also present in the mixture [200]. Anticoagulant oligosaccharides contain a unique pentasaccharide sequence responsible for the anticoagulant effects of heparins [44]. In recent years, attention has shifted to the non-anticoagulant activities of heparins [46]. Importantly, many studies have shown that the non-anticoagulant effects of heparins, such as anti-inflammatory activities, are independent of their anticoagulant activity and largely dependent on the presence of non-anticoagulant oligosaccharides [46,142]. A number of

studies have investigated the effects of heparins on angiogenesis [201]. Interestingly, it has also been observed that UFH has a contrasting effect; some of the chemically synthesised oligosaccharides of heparins have an anti-angiogenic effect, while some have a pro-angiogenic effect and importantly these effects are dependent on the size and sulphation patterns of oligosaccharides [201-203].

The ability of heparins to modulate cellular growth has largely been studied with intact heparins (mixture of oligosaccharides) [190,194,198,199] instead of their individual unmodified oligosaccharides. We hypothesised that intact heparins are responsible for either anti- or pro-proliferative effects depending on the types of non-anticoagulant oligosaccharides present in the heterogeneous mixture of heparins. Hence, the present investigation examined and compared the cellular growth modulation effects of intact UFH, intact LMWHs (namely enoxaparin and dalteparin) and structurally unmodified oligosaccharides of enoxaparin on several human intestinal epithelial cell lines.

2.2 Materials and methods

Human colon carcinoma epithelial cell lines (HCT-116, HT-29 and DLD-1) were obtained from European Collection of Cell Cultures (ECACC) and cultured under standard conditions (37°C, 5% CO₂, 95% humidity) in modified McCoy's 5A medium or Roswell Park Memorial Institute (RPMI) 1640 medium. All culture media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% v/v heat inactivated foetal calf serum (FCS, Gibco, Life Technologies Victoria, Australia). When cells had reached confluence, they were washed twice with phosphate buffered saline (PBS) and followed by 1mM ethylenediaminetetraacetic acid (EDTA) solution before being harvested using trypsin (0.5 g/L)/ EDTA (0.2 g/L). Cells were routinely passaged twice a week and seeded at concentrations of 2 or 3 ×10⁴ cells /cm². All chemicals and supplies were obtained from

Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise specified. All cell culture plastics were obtained from Corning (Tewksbury, MA, USA) unless otherwise specified.

2.2.1 Foetal calf serum mitogenic assay

To assess the mitogenic effect of FCS on the intestinal epithelial cell line (HCT-116), 1500 or 2500 HCT-116 cells/ 100 μ l/ well were seeded in 96 well-plates (flat bottom) in McCoy's 5A media containing 5% v/v FCS overnight. The media was removed after 24 hours and replaced with media containing FCS at 0.1%, 0.5%, 1%, 2% or 10%. Five parallel wells were used for each concentration of FCS in a single experiment. At 24, 48 and 72 hours after the media change, cell proliferation was indirectly assessed by measuring metabolically active cells using a commercially available water-soluble tetrazolium dye, WST-1 reagent (Roche, Mannheim, Germany).

2.2.2 Basic fibroblast growth factor mitogenic assay

To test the mitogenic capacity of basic fibroblast growth factor (bFGF), 2500 HCT-116 cells/ 100 μ l/ well were seeded in 96 well-plates in McCoy's 5A media containing 5% FCS overnight. The culture media was removed after 24 hours and replaced with fresh media containing 5% v/v FCS and 0, 0.1, 1 or 10 nM bFGF respectively. At 24, 48, 72 and 96 hours, cell proliferation was measured using WST-1 reagent. Alternatively, 1500 or 2500 HCT-116 cells/ 100 μ l/ well were seeded and incubated overnight before a media change. Cells were incubated for the following 24 hours in the media with reduced FCS (0.1%, 0.5% or 1.0% v/v) before the addition of 10 μ l of bFGF to achieve final concentration of 0.1 nM bFGF. PBS and FCS (10% v/v) were included as negative and positive controls respectively. Cell proliferation was measured using WST-1 reagent at -24, 0, 24 and 48 hours. All

treatment conditions were analysed in five parallel wells and means of absorbance plotted from one of the two similar experiments.

2.2.3 Quantification of cell proliferation using WST-1 reagent

Cell proliferation was indirectly assessed by quantifying metabolically active cells using WST-1. For this purpose, 50 μ l of growth media were discarded from each well before 5 μ l of WST-1/ well was added and incubated for 4 hours. The absorbance of the formazan dye in solution was measured at 450 nm (Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer, SkanIt Software). The reference wavelength is set at 690 nm. Background values from cell-free media only wells incubated with dye were obtained. Cell proliferation was presented as average of absorbance (A , $A_{450\text{nm}} - A_{690\text{nm}}$) or expressed as fold change of control and standard deviation.

2.2.4 UFH and LMWHs

Heparin (DBL heparin sodium, Hospira, 5000 IU/ 0.2 ml, referred to unfractionated heparin (UFH) in the text), enoxaparin (Clexane, Sanofi Aventis, 20 mg/ 0.2 ml) and dalteparin (Fragmin, Pfizer, 2500 IU/ 0.2 ml) were diluted with phosphate buffer saline (PBS) when necessary to achieve final concentrations of 0 (for control), 0.01, 0.1, 1, 10, 100, 200, 400, 1000 μ g/ml (with 160 IU = 1 mg heparin, 100 IU = 1 mg enoxaparin and 156.25 IU = 1 mg dalteparin).

2.2.5 Isolation of enoxaparin oligosaccharides

2.2.5.1 HPLC instrumentation

To isolate individual enoxaparin oligosaccharides, a high performance liquid chromatography (HPLC) system consisting of a Dionex UltiMate 3000 pump, Dionex

UltiMate 3000 auto sampler, Dionex UltiMate 3000 RS Column compartment and Dionex UltiMate 3000 Variable Wavelength detector (Thermo Scientific, NSW, Australia) was used. Data acquisition and instrument control were carried out using Chromeleon[®] software.

2.2.5.2 Ion-exchange chromatography

The separation of enoxaparin was carried out using a previously described ion-exchange chromatography (IC) method [76]. IC was performed using a Carbo PacTM PA-100 (250 × 9 mm × 8.5 µm) anion ion-exchange column (Thermo Scientific, NSW, Australia). The mobile phases consisted of 2 M NaCl (A) and Milli-Q water (B). The mobile phase was filtered using a Millipore vacuum equipped with a 0.45 µm filter. The filtered mobile phase was degassed using an ultrasonic bath (Sonorex RK-100, Bendelin Electronic, Germany) for 10 minutes prior to its use. The separation of enoxaparin was achieved using a linear gradient elution of mobile phase A from 32% to 74% over 70 minutes. Then the column was re-equilibrated with 32% of mobile phase A for 15 minutes. The flow rate was maintained at 2 ml/ minute. UV detection was carried out at 232 nm. A total of 250 µl enoxaparin (50 mg/ml) was loaded into the column for each injection of sample.

2.2.5.3 Collection and desalting of IC separated enoxaparin oligosaccharides

The collection and desalting of IC separated enoxaparin oligosaccharides was carried out as described previously [76]. A total of sixteen runs (n = 16) of enoxaparin separation were carried out to collect sufficient amount of oligosaccharides for assay. Briefly, for each enoxaparin run, fourteen (14) oligosaccharides of enoxaparin (1.5 - 33 ml each) were isolated and collected over the same elution period between 10.0 and 65.0 minutes in 15 ml tubes. The IC collected samples contain NaCl, water and the enoxaparin oligosaccharides. The samples were concentrated using a miVac DNA centrifugal concentrator (Genevac Ltd,

Suffolk, UK) at 50 °C until the formation of salt crystals. The salt was subsequently removed using methanol precipitation. For this purpose, to each sample containing salt crystal, approximately 12 ml of 80% v/v anhydrous methanol was added and vortexed to dissolve crystallized salt in the solvent. The solution was then centrifuged (10 minutes, 3000 rpm, GLC-2, Sorvall, DJB Labcare, Newport Pagnell, UK) before the supernatant containing NaCl was carefully removed, leaving the precipitated oligosaccharides behind. The desalting step was repeated until all crystallized salt was fully removed to attain precipitated oligosaccharides. To ensure oligosaccharides were fully precipitated, the samples were kept at 4 °C overnight. The supernatant was carefully discarded the following day and traces of methanol were removed using the miVac DNA concentrator (Genevac Ltd, Suffolk, UK). The remaining precipitant of each centrifuge tube was dissolved in 500 µl of Milli-Q water. Approximately 4 ml of the same oligosaccharides of enoxaparin were then combined into a single tube and the volume was reduced to 1 ml each by concentration. The samples were then stored at 4 °C until use. The concentration of each enoxaparin oligosaccharide was calculated using the peak areas of desalted oligosaccharides. These oligosaccharides were tested for their effects on colony formation at 100 µg/ml. The effects of oligosaccharides 2 and 7 on cell cycle were also tested.

2.2.6 Cell growth assay

A total of 2500 HCT-116 cells were seeded in 12 well-plates using McCoy's 5A culture media containing either 0.5% or 10% FCS respectively. Final concentrations of heparin, enoxaparin, dalteparin (as indicated: 0.01 - 400 µg/ml) were added into wells in triplicate. Cell growth was followed over a maximum period of two weeks with media replacement bi-weekly before the cells were stained with trypan blue or alternatively protein quantification. For cell staining in 12 well-plates, cells were fixed with ice cold methanol at - 20 °C for 10

minutes before being incubated with 0.4% w/v trypan blue for 5 minutes. Cells were washed twice with tap water and air-dried overnight. Images of 12-well plate from one of the three similar experiments was taken with a Canon trans-illuminant flatbed scanner.

2.2.7 Protein quantification

Protein levels were measured using the Bio-Rad detergent-compatible protein assay (BioRad Laboratories, Gladesville, NSW, Australia) according to the manufacturers protocol. Briefly, bovine serum albumin (BSA) standards were prepared in lysis buffer as serial dilutions between 0.08 and 5 mg/ml. Cells were washed with PBS twice before being lysed in 100 µl/well of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Tween 20 and 0.2% Triton X-100) for 15 minutes at 4 °C. The lysate of each well was centrifuged in Eppendorf tubes (10 minutes, 14000 rpm, 4 °C, Eppendorf® Micro Centrifuge, 5417R, Eppendorf, NY, USA). For measuring protein content, 10 µl of supernatant or protein standards were pipetted in triplicate into a 96 well-plates before 25 µl of reagent AS (alkaline copper tartrate and surfactant) and 200 µl of reagent B (diluted Folin) were added into each well. After 15 minutes, absorbance was read at 750 nm (Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer, SkanIt Software). Protein concentrations were calculated using the standard curve derived from the BSA standards. One representative protein content of each condition compared with control from three independent experiments was demonstrated.

2.2.8 Colony formation assay

Cells (HCT-116: 200, 500, 800, 1000 or 2000 cells/ dish; DLD-1: 800 cells/ dish, HT-29: 800 cells/ dish) in 10 ml of RPMI 1640 or McCoy's 5A media containing FCS (0.5%, 2%, 5% or 10%) were seeded into 10 cm tissue culture dishes (Nunc, Thermo Fisher Scientific,

Victoria, Australia) to allow overnight adherence without drug interference. Cells were treated in four replicate dishes per condition for 2 individual experiments with 10 - 50 μ l of heparin, enoxaparin and dalteparin to achieve required final concentrations (0.01 - 400 μ g/ml) or 61 - 266 μ l of enoxaparin oligosaccharides for 100 μ g/ml in a single experiment. The cells were incubated for two weeks without media change before being washed twice with PBS and fixed with 2% w/v paraformaldehyde in PBS for 15 minutes at room temperature. Subsequently, colonies were stained with 1 ml of 0.4% w/v trypan blue or 0.1% w/v crystal violet (Oxoid, West Heidelberg, VIC, Australia) for 5 minutes before washed twice with tap water and air-dried. Colonies consisting of more than 50 cells were manually scored using a standard light microscope (Inverted Microscope, Model INV-100, Aktivlab, SA, Australia). Colony formation was expressed as the number of colonies or as the percentage of colonies compared to control values.

2.2.9 Cell viability assays

2.2.9.1 Colorimetric assay

A total 2500 HCT-116 cells/ 100 μ l/ well were seeded in 96 well-plates in McCoy's 5A media containing 5% v/v FCS overnight before the FCS concentration was reduced to 0.5% v/v by media change. After 24 hours, 10 μ l of heparin, enoxaparin and dalteparin were added into each well to achieve concentrations of 0.01 - 1000 μ g/ml. PBS and 10 % v/v FCS were included as negative and positive controls respectively. For all experiments at least five parallel wells were used for a single condition. After 24 hours incubation with and without treatment, cell proliferation was measured using WST-1 reagent as above. Cell proliferation in the treatment groups was presented as fold-change of absorbance relative to the negative control, the average of four to five independent experiments.

2.2.9.2 Assessment of cell viability using trypan blue exclusion assay

Briefly, 5000 cells (HCT-116, DLD-1 and HT-29)/ well were seeded into 24-well-plates overnight in McCoy's 5A or RPMI 1640 media containing 2% v/v FCS before incubation with heparin, enoxaparin or dalteparin at 400 µg/ml in four replicate wells. Cells were harvested by trypsinization on day 5 after the start of treatment, collected by centrifugation (5 minutes, 300 g, centrifuge CM-6MT, Elmi Ltd, Riga, Latvia) before being resuspended in 100 µl of culture media and 100 µl of 0.4% w/v trypan blue solution. Healthy (no staining) and damaged cells (nucleus stained blue) were scored using a Neubauer Chamber. Briefly, resuspended cells were allowed to diffuse beneath the coverslip. By using light inverted microscope, the number of cells in the 25 central squares of the grid was counted. Total cell counts per area were recorded for each condition. Cell viability was expressed as the percentage of damaged to viable cells, presented as mean from quadruplets of one of the three similar experiments.

2.2.9.3 Detection of apoptotic cells

Apoptosis was measured using a commercially available kit (Annexin V-FITC Apoptosis Detection Kit; BD Pharmingen). Briefly, 2×10^4 HCT-116 cells were seeded into T-25 flasks overnight in McCoy's 5A media containing 2% v/v FCS before being incubated with final concentrations of heparin or enoxaparin at 400 µg/ml. After four days of treatment, cells were harvested by trypsin, washed with PBS twice. Cells were resuspended in 100 µl of binding buffer in a 5 ml round bottom FACS tube (BD Falcon, BD Biosciences, NSW, Australia). To each tube, 5 µl of fluorescein isothiocyanate (FITC) conjugated annexin-V and 5 µl of propidium iodide (PI; 50 µg/ml) were added and incubated in the dark at room temperature for 15 minutes. After incubation, 400 µl of binding buffer were added before cells were analysed by flow cytometer (FACSCanto, Becton Dickinson, CA, USA) to

differentiate apoptotic cells (annexin V-FITC positive and PI negative, lower right quadrant) from necrotic cells (annexin V-FITC and PI positive, upper right quadrant). Five thousand events were collected from each sample (FACS Diva software, Becton Dickinson) and analysed (FlowJo software, version 10; Treestar, Ashland, USA). Samples were analysed in triplicates in a single experiment.

2.2.10 Cell cycle analysis

Cell cycle analysis was carried out using PI staining and flow cytometry. HCT-116 cells (4×10^5) were seeded in 6 well-plates and allowed to adhere for 7 hours followed by treatment with heparin, enoxaparin, dalteparin or enoxaparin oligosaccharides in triplicate per condition at the concentration as indicated: 100 $\mu\text{g/ml}$ or 400 $\mu\text{g/ml}$. Three independent experiments were performed for heparins whereas a single experiment was performed for enoxaparin oligosaccharides. After 18 hours of drug exposure, cells were harvested by trypsinization and washed with PBS before being resuspended in 750 μl of PBS. Three millilitres of ice cold 95% ethanol was added steadily to the cells while vortexing and the cells were fixed overnight at 4 $^{\circ}\text{C}$. Subsequently, the cells were washed with PBS twice before being resuspended in 500 μl of PBS and counted to adjust the cell density to 1×10^6 cells/ ml. The cell solution from each sample was transferred into a 5 ml round bottom FACS tube and treated with 5 μl of Ribonuclease A (10 mg/ml) (Sigma-Aldrich, Castle Hill, NSW, Australia) before the cellular DNA was labelled with 50 μl of PI (1 mg/ml) and incubated in dark in water bath at 37 $^{\circ}\text{C}$ for 15 minutes. Fluorescence was measured using a flow cytometer (FACSCanto, Becton Dickinson, CA, USA). Ten thousand events per sample were collected in a list mode with the accompanying software (FACS Diva software, Becton Dickinson). Effects on the cell cycle were determined by changes in percentage of cell distribution at each phase of the cell cycle, and assessed by histograms (FlowJo software,

version10; Treestar, Ashland, USA). The proportion of cells in sub-G₁, G₁, S and G₂ phase was quantified and presented as percentage in each phase from total cells.

2.2.11 Statistical analysis

All data are presented as mean \pm standard deviation (SD) or as percent change compared to control. Using GraphPad Prism (version 6, GraphPad Software Inc, CA, USA), statistical significance was evaluated using Student's t-test and one way analysis of variance (ANOVA), where appropriate, followed by Dunnett's multiple comparisons tests to evaluate the difference between treatment groups and control. A *p* value of < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Serum-dependent cell growth

In order to investigate the proliferative capacity of human colonic epithelial cells in response to a growth factor or heparins, we first assessed the basal growth rate of human colonic epithelial cells in the presence of different concentrations of FCS (0.1% to 10% v/v). The proliferation of HCT-116 cells was reflected by increased absorbance, which reflects increasing numbers of metabolically active cells (Fig. 2.1). FCS had a dose-dependent growth stimulating effect on cell proliferation based on the abundance of different growth factors and nutrients. From 24 to 72 hours, the absorbance signal increased from approximately 0.2 to 1.5 as a function of serum concentration (Fig. 2.1A & Fig. 2.1B). The signal was the lowest when FCS was restricted at 0.5% or 0.1% compared to higher concentrations of FCS. At 24 and 48 hours, the signals obtained from 2500 cells/ well (Fig. 2.1B) were consistently higher than 1500 cells/ well (Fig. 2.1A) irrespective to any FCS concentrations. Similarly, a faster cell growth was seen over the period of 24 to 48 hours for

2500 cells/ well; the growth rate became slower from 48 to 72 hours for the conditions with FCS at 1, 2, 5 or 10% v/v (Fig. 2.1B). On the other hand, the growth rate of 1500 cells/ well increased linearly from 24 to 72 hours for all FCS concentrations above 1% (Fig. 2.1A).

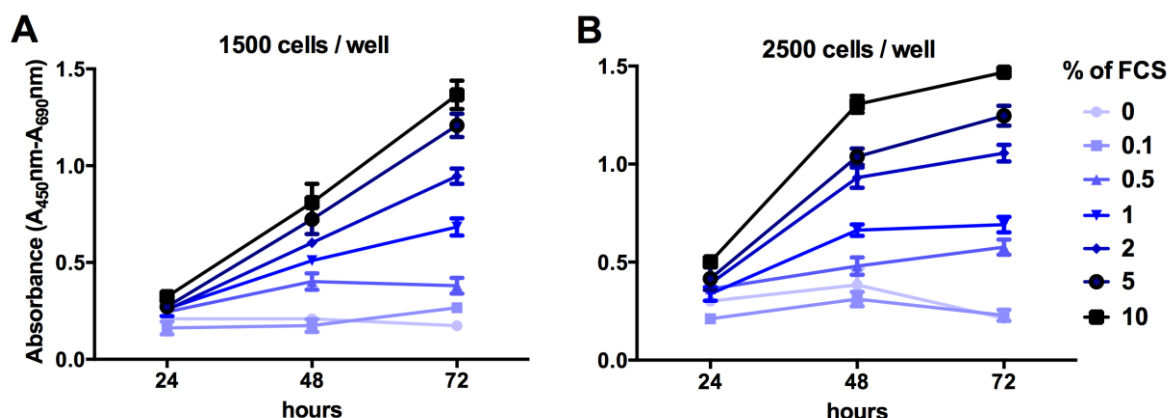


Figure 2.1 FCS-dependent epithelial cell growth.

HCT-116 cell proliferation at 10%, 5%, 2% and 0.5, 0.1, 0% of FCS with seeding density at (A) 1500 cells/ well and (B) 2500 cells/ well. Data represents the mean of five individual samples per treatment of one typical experiment. Error bars depict standard deviations.

2.3.2 Effect of basic fibroblast growth factor on HCT-116 cells

Previously, human colonic epithelial cells, HCT-116, were reported to respond to basic fibroblast growth factor (bFGF) stimulation in culture media containing 5% of FCS [57]. We aimed to replicate the results to investigate the effect of bFGF on cell proliferation before studying the cellular proliferation response in the presence of heparins. However, when replicating the previous described conditions, we did not detect any significant mitogenic effect of bFGF. Compared to untreated control, the absorbance signal of treated wells did not significantly differ at any time points from untreated wells and reached a plateau after approximately 72 hours (Fig. 2.2A). Furthermore, there was also no indication of the reported dose-dependency of bFGF in 5% FCS containing media detectable.

Since high concentrations of growth factors contained in FCS (5%) could likely have prevented the mitogenic effect of additional bFGF supplementation, we reduced the concentration of FCS in the culture medium and cells were conditioned for 24 hours before cells were exposed to 0.1 nM bFGF, which was reportedly an optimal concentration to stimulate cell growth [57,204]. At a cell density of 2500 cells/ well in media containing 1% of FCS, no significant differences were seen at 24 and 48 hours in the presence of 0.1 nM bFGF compared to untreated control (Fig. 2.2B). Even at a lower cell density (1500 cells/ well) and reduced FCS concentrations (0.5% v/v, Fig. 2.2C and 0.1% v/v, Fig. 2.2D), absorbance increased steadily but the differences between the bFGF-treated and untreated were not significant at any point. In media containing 0.1% FCS, where cells responded well to FCS (10%), bFGF resulted in only a 10.2% increase in absorbance after 24 hours compared to untreated control (Fig. 2.2D). Due to these disappointing results, we stopped investigating the effects of bFGF on cell growth and thus abandoned the study on possible interactions between bFGF and heparins.

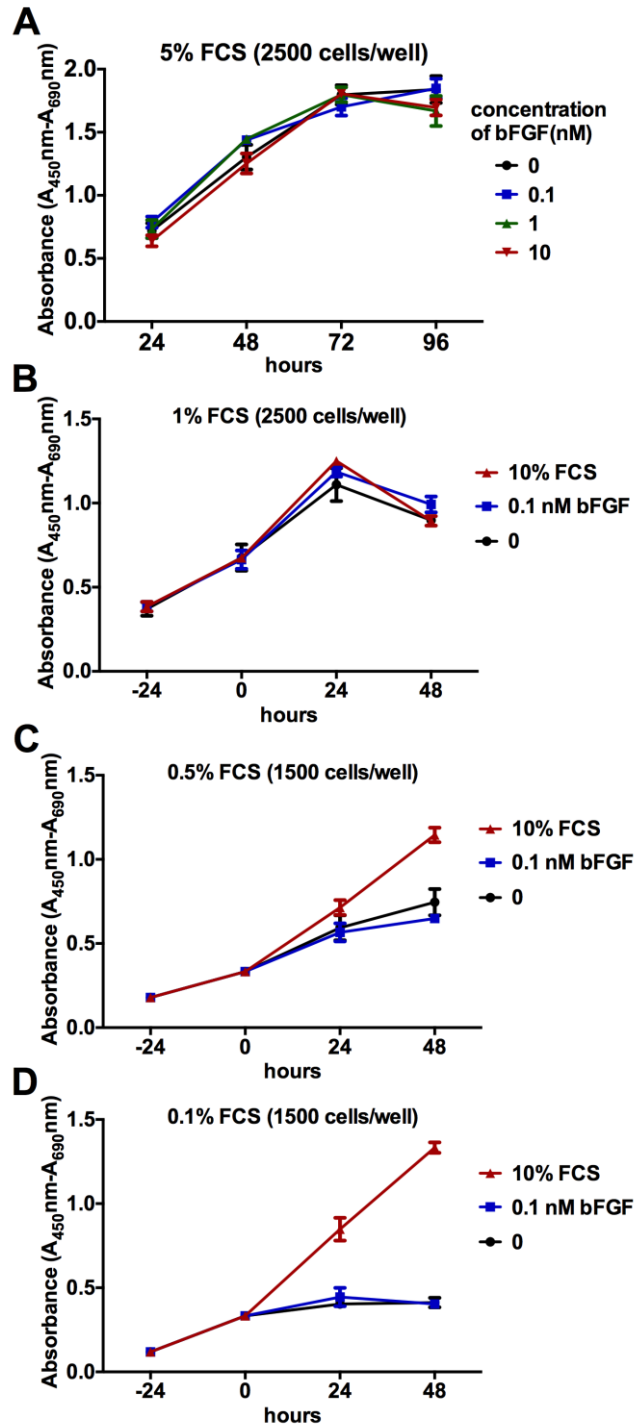


Figure 2.2 Epithelial cell growth with bFGF.

(A) HCT-116 cell proliferation in the presence of 0.1, 1 and 10 nM of basic fibroblast growth factor (bFGF) in culture media containing 5% of FCS. HCT-116 cell proliferation in the absence and presence of 0.1 nM of bFGF in culture media containing (B) 1%, (C) 0.5%, (D) 0.1% of FCS. FCS was added to media to a final concentration of 10% v/v FCS as positive control. Data represents the mean of five individual samples per treatment of one typical experiment. Error bars depict standard deviations.

2.3.3 Serum dependency of the effect of heparins on cell growth

Since heparins were previously reported to inhibit cell proliferation [184], we attempted to compare UFH efficacy against enoxaparin under normal growth conditions (10% v/v FCS) with the same cell line. Contrary to previous report [194,205,206], no reduction of cell growth by UFH or enoxaparin in HCT-116 cells was detected using colony formation as readout. There was also no indication of a dose dependency under these conditions (Fig. 2.3A) that was reported earlier [205]. To investigate if the abundance of growth factors in the serum could mask any effects of UFH in our cell culture, the colony formation assay was repeated in the absence or presence of UFH at different FCS concentrations (Fig. 2.3B). Under these conditions, UFH clearly showed a significant reduction ($p = 0.0053$) of colony numbers at 5% FCS (58.2% of control) and lead to a strong trend (74.2% of control) at 2% FCS. While at 10% FCS concentration no difference in colony numbers was detected, at 0.5% the lack of colonies prevented any analysis (Fig. 2.3B).

This serum concentration dependency was also observed when we investigated cell growth in a mass culture at 0.5% FCS concentration (Fig. 2.3C). Under these conditions, HCT-116 cells were clearly able to proliferate in the absence of heparins. However, in the presence of all heparins, a significant reduction in cell density could be observed. In agreement with the results above, no treatment-induced difference in cell density was evident at 10% FCS (Fig. 2.3C). Consequently, for all further experiments, FCS concentrations of 0.5% to 2% were used. The observed reduction in cell density was quantified by measuring protein content per well and again UFH concentrations between 100 and 400 ug/ml significantly ($p < 0.001$) reduced protein content per well dose-dependently by up to 60.8% of control at the highest UFH concentration (Fig. 2.3D).

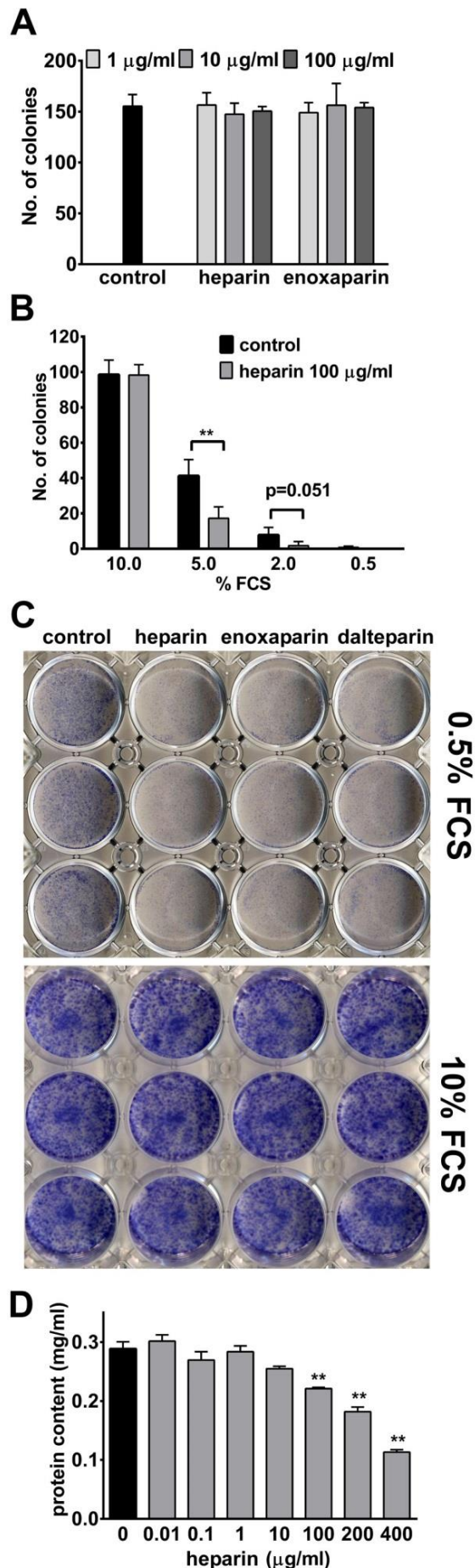


Figure 2.3 Effect of heparins on epithelial cell growth.

(A) Colony formation of HCT-116 cells in the presence of 10% of FCS with exposure to three different concentration of heparin or enoxaparin. Data represents the mean of four individual samples per concentration of one typical experiment. Error bars depict standard deviations.

(B) Colony formation of HCT-116 cells at 10%, 5%, 2% and 0.5% of FCS with exposure to 100 µg/ml of heparin. Data represents the mean of four individual samples per concentration of a typical experiment. Error bars depict standard deviations.

** $p < 0.01$ versus control. (C) HCT-116 cells were grown in the absence (control) or presence of 100 µg/ml of heparin, enoxaparin and dalteparin respectively, before fixed and stained with trypan blue. (D) HCT-116 cells were grown in media containing 0.5% v/v FCS and heparin (0.01 - 400 µg/ml) for 14 days before protein content / well was measured. Data represents the mean of triplicates from a representative experiment. Error bars depict standard deviations. ** $p < 0.01$ versus control.

2.3.4 Dose-dependent reduction of colony formation by heparins

To compare a possible dose-dependent reduction of proliferation by UFH against LMWHs, concentrations ranging from 0.01 to 400 $\mu\text{g/mL}$ were tested in the colony formation assay (Fig. 2.4A-C). UFH and the two LMWHs, enoxaparin and dalteparin, dose-dependently reduced colony formation by up to 34.9%, 44.5% and 33.0%, respectively. Interestingly, significant reductions in colony numbers were detected at 10 $\mu\text{g/ml}$ for enoxaparin (Fig. 2.4B), while significance was achieved for UFH (Fig. 2.4A) and dalteparin (Fig. 2.4C) only at concentrations at and above 100 $\mu\text{g/ml}$. This effect was clearly not cell line specific since UFH and LMWHs induced comparable reductions in colony numbers in 3 different intestinal cancer cell lines (HCT-116, DLD-1, HT-29) used (Fig. 2.4D).

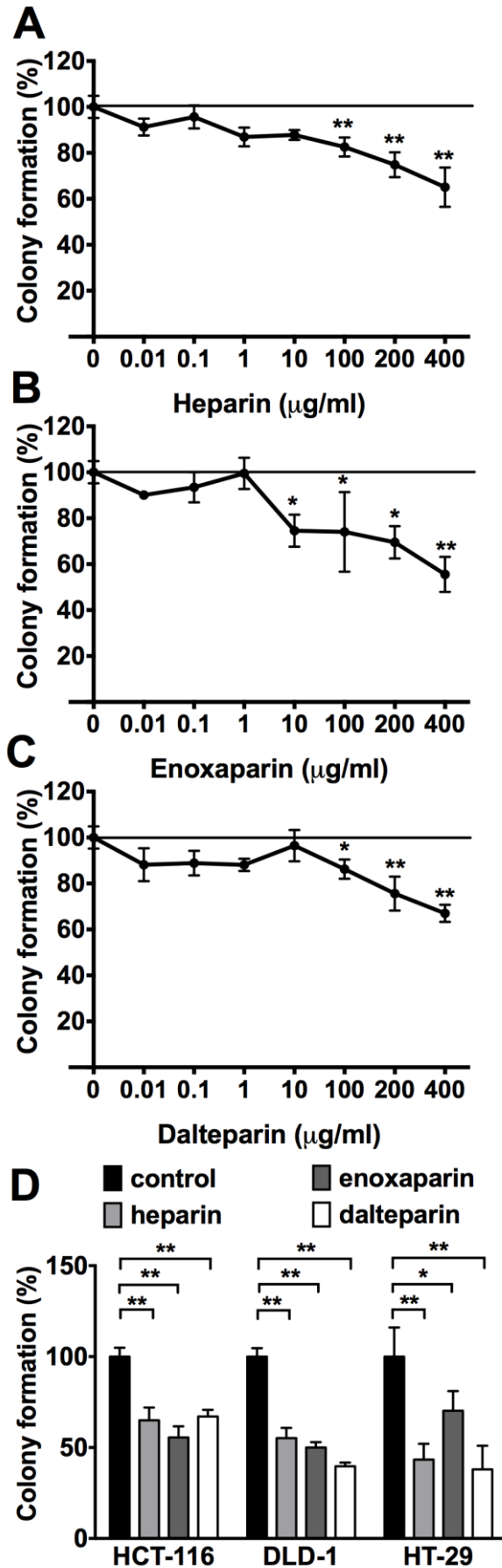


Figure 2.4 Inhibition cellular colony formation by heparins.

Colony formation of HCT-116 cells in the presence of (A) heparin, (B) enoxaparin, (C) dalteparin and 2% v/v FCS. (D) Epithelial cells (HCT-116, DLD-1, HT-29) were exposed to 400 $\mu\text{g/ml}$ in media containing 2% v/v FCS. Data represents the average of four individual samples per concentration of a typical experiment. Error bars depict standard deviations. * $p < 0.05$, ** $p < 0.01$ versus control.

2.3.5 Inhibition of cell cycle progression by heparins

In order to explain the observed reduction of colony numbers by heparins, their effect on cell cycle progression was analysed (Fig. 2.5). Consistent with the results described above, after 18 hours of treatment both UFH and LMWHs significantly increased the proportion of cells in G₁ phase and at the same time decreased cell numbers in S and G₂ phase (Fig. 2.5A and 2.5B).

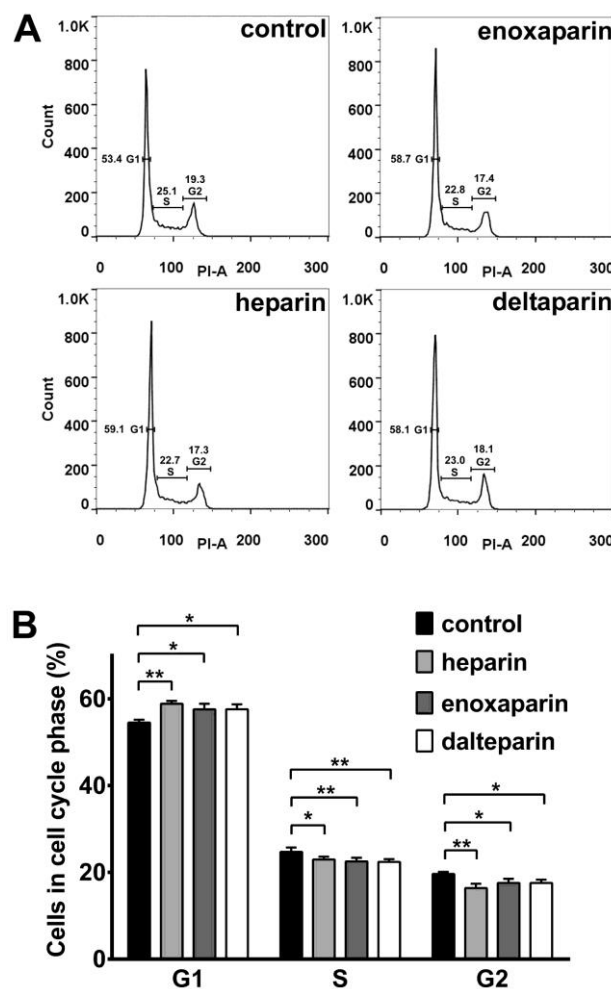


Figure 2.5 Inhibition of cell cycle progression by heparins.

Flow cytometric cell cycle analysis of HCT-116 cells after 18 hours exposure to heparin, enoxaparin, dalteparin at 400 μ g/ml in media containing 2% of FCS. (A) Representative histograms of cell counts versus propidium iodide (PI) for control versus treatment groups. (B) Graphical representation of the percentage of cells in G₁, S, G₂ phases determined by gating. Data represents the mean of three individual samples per treatment of a typical experiment. Error bars depict standard deviations. * $p < 0.05$, ** $p < 0.01$ versus control.

2.3.6 Effect of heparins on cellular viability

Although the observed effects on cell cycle progression alone could explain the reduced colony numbers described above, heparins have also been reported to induce cell death [207]. To investigate if cell death was also present in this experimental system, cellular viability was measured by the commonly used redox-dye WST-1 (Fig. 2.6A). In HCT-116 cells, after 24 h of treatment with UFH and LMWHs, no signs of cell death could be detected even at concentrations up to 1 mg/ml (Fig. 2.6A). A second assay based on trypan blue exclusion was employed to detect possible delayed toxicities. Consistent with the prior results, this approach also failed to demonstrate any significant toxicity of heparins after 5 days of treatment in 3 different cell lines (Fig. 2.6B). Finally, Annexin V-FITC combined with PI staining was performed to detect the possible presence of apoptotic and/or necrotic cells (Fig. 2.6C). After treatment of HCT-116 cells with UFH or enoxaparin, no increased numbers of apoptotic (quadrants Q2 and Q3) or necrotic cells (quadrants Q1 and Q2) could be detected (Fig. 2.6C).

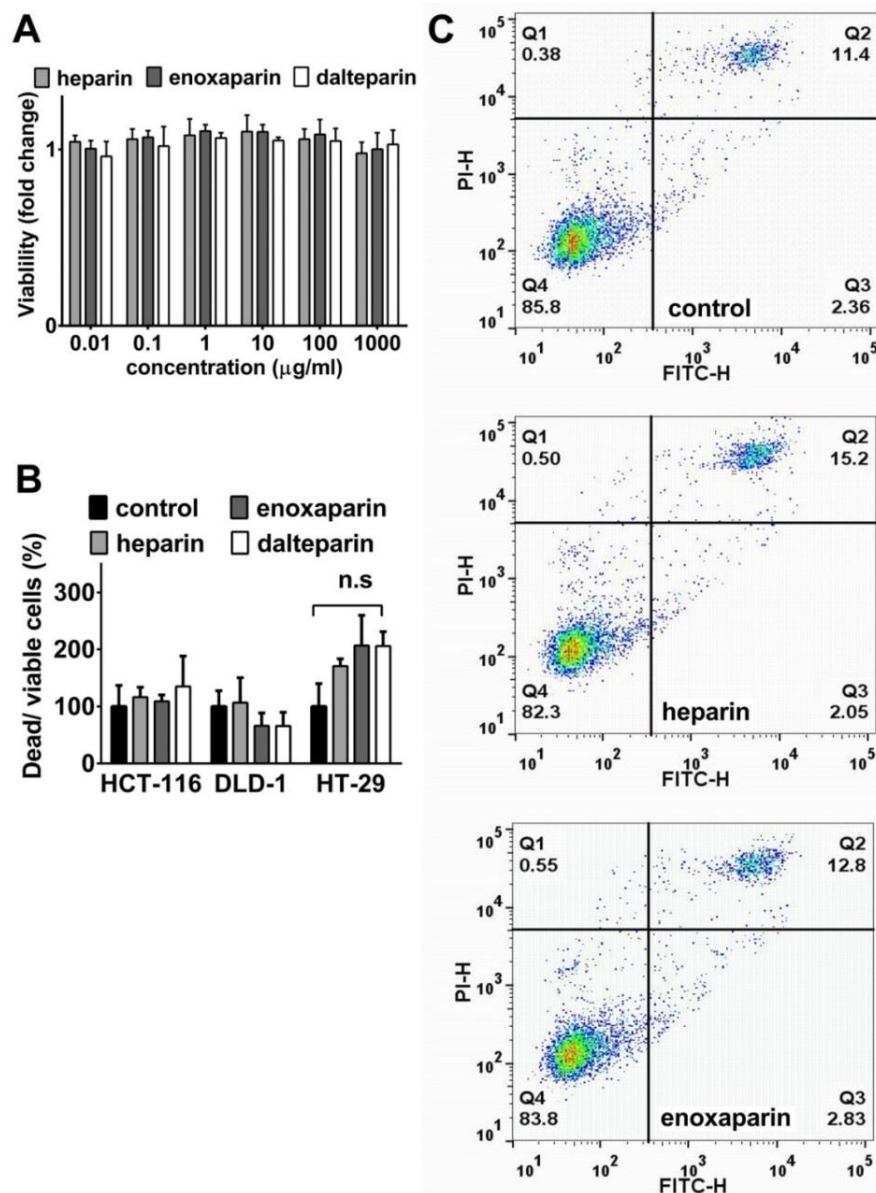


Figure 2.6 Effect of heparins on cellular viability.

(A) HCT-116 cells were treated with heparin, enoxaparin, dalteparin (0.01-1000 $\mu\text{g/ml}$) for 24 hours before the cell viability was measured using WST-1. The fold change of absorbance signal in negative control was designated as 1. This data represents the average of five replicates from four independent experiments. Error bars depict standard deviations. (B) Epithelial cells (HCT-116, DLD-1, HT-29) were incubated with heparin, enoxaparin, dalteparin at 400 $\mu\text{g/ml}$ in media containing 2% of FCS in for over 5 days. The cell viability was determined by trypan blue dye exclusion assay where viable cells without staining blue while non-viable cells appearing blue were counted. Error bars depict standard deviations. (C) HCT-116 cells were untreated or treated with heparin and enoxaparin at 400 $\mu\text{g/ml}$ in media containing 2% of FCS. After 96 hours, Annexin V-FITC/ PI staining was analysed by flow cytometry. Data represents samples of control, heparin and enoxaparin of a typical experiment.

2.3.7 Differential effects of enoxaparin oligosaccharides on proliferation and cell cycle

Heparins are a complex mixture of oligosaccharides of different lengths or sizes, various degrees and distribution of sulphate groups and sulphation patterns with and without anticoagulant activity [208]. We therefore investigated if different oligosaccharides might harbour different activities with regards to the proliferative capacity of cells as observed in the previous results. For this purpose, enoxaparin was separated into 14 different oligosaccharides using IC based on size and charge (Fig. 2.7A). Enoxaparin is a polydisperse mixture of highly sulphated oligosaccharides with molecular weights or sizes from dp2 (two saccharides ~600 Da) to dp24 (twenty four disaccharides ~ 8000 Da). As the oligosaccharides increase in size, so does the degree of sulphation. Based on this chemical nature of oligosaccharides, larger oligosaccharides are retained longer or eluted later in the columns during the IC salt gradient separation as the sulphate groups interact with the oppositely charged stationary phase. The collected intact oligosaccharides from IC are highly polar and soluble in water. The retention time of enoxaparin oligosaccharides were as shown in Figures 2.7 (A).

IC allowed a maximum of 12.5 mg of enoxaparin/ 250 μ l to be loaded onto a semi-preparative CarboPac PA100 column with each injection. Compared to analytical column, this semi-preparative column facilitated the collection of sufficient quantities of oligosaccharides for the investigation of their non-anticoagulant activities after 16 runs. For example, the concentrations of IC-derived oligosaccharide 2 (dp2, two saccharides) and oligosaccharide 7 (dp6, six saccharides) after desalting were found to be approximately 5.69 mg/ml and 6.95 mg/ml respectively, with end volume of 1 ml each. These concentrations were sufficient to investigate the proliferative activities of each oligosaccharide at 100

µg/ml. The concentration of each desalted oligosaccharides was calculated using the differences in the peak areas of oligosaccharides and oligosaccharides of enoxaparin at known concentrations eluted at the same time. The average recoveries of enoxaparin oligosaccharides were found to be 80% [76].

When these oligosaccharides were individually assessed for their effect on colony formation, distinct profiles were observed (Fig. 2.7B). While some oligosaccharides (2 and 11), potently and significantly reduced colony formation, others (6, 7, 8, 10, and 12) surprisingly increased colony numbers. It has to be noted that under these conditions, the intact enoxaparin, which contains all oligosaccharides, inhibited colony formation dose-dependently (grey bars) as described above (Fig. 2.7B). Based on the differential effects on proliferation and also their respectively no and low inherited anticoagulant activity [76], oligosaccharides 2 and 7 were selected for comparison,. This differential effect of 2 selected exemplary oligosaccharides could also be demonstrated when looking at cell growth in a mass culture (Fig. 2.7C). While oligosaccharide 2 clearly reduced cell density more potently compared to intact enoxaparin at the same concentration, oligosaccharide 7 resulted in a strong increase in cell density. Finally, these two oligosaccharides were also tested for their effects on cell cycle progression (Fig. 2.7D). Consistent with the results from the colony formation assay, oligosaccharide 2 strongly increased the number of cells in G₁ phase while leading to a significant reduction in S and G₂ phase cells (Fig. 2.7D). It is important to note that at the same concentration, this effect was more pronounced for oligosaccharide 2 compared to intact enoxaparin (Fig. 2.7D). In line with the previous data, oligosaccharide 7 significantly reduced the number of cells in G₁, while effects on S and G₂ phase did not reach statistical significance. There was no difference of sub-G₁ peaks of treated cells compared to control (data not shown).

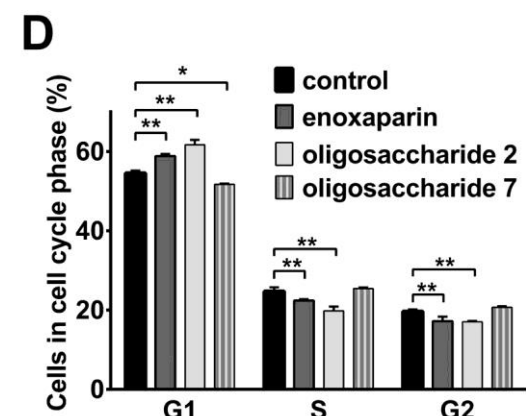
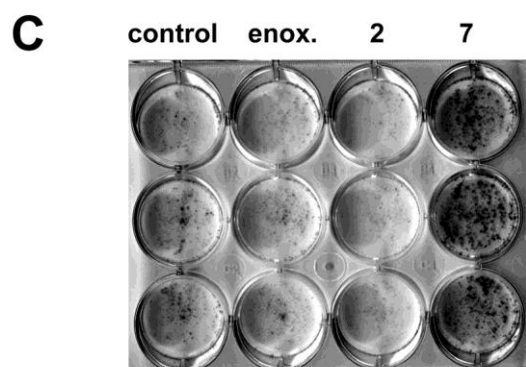
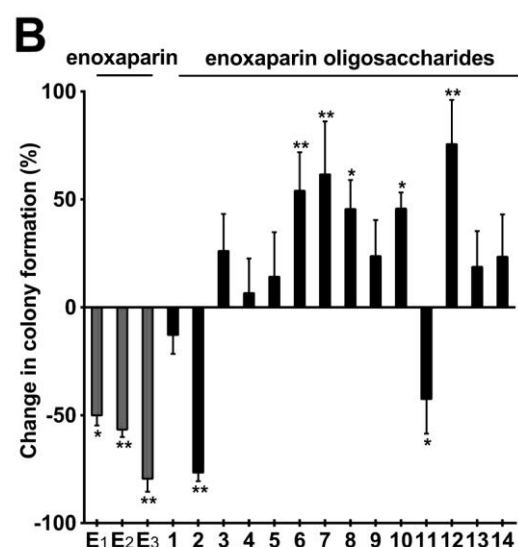
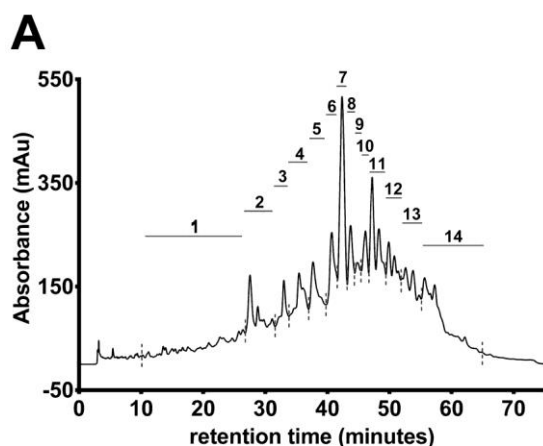


Figure 2.7 Differential effects of enoxaparin oligosaccharides on proliferation and cell cycle.

(A) Ion-exchange chromatographic separation of enoxaparin oligosaccharides. Numbers and lines indicate enoxaparin oligosaccharide tested. (B) Effect of enoxaparin (E1: 10 $\mu\text{g/ml}$; E2: 100 $\mu\text{g/ml}$, E3: 200 $\mu\text{g/ml}$) and its oligosaccharides (1-14: 100 $\mu\text{g/ml}$) on HCT-116 epithelial cell colony forming capacity in media containing 2% of FCS. The total colony formations were enumerated at the end of experiment and presented as percentage change versus negative control. This data represents the average of four individual samples per concentration of a typical experiment. Error bars depict standard deviations. * $p < 0.05$, ** $p < 0.01$ versus negative control. (C) HCT-116 cells were incubated in the absence (control) or presence of 100 $\mu\text{g/ml}$ of enoxaparin (enox.) or its oligosaccharides (2 and 7) respectively before fixed and stained with trypan blue. (D) Flow cytometric cell cycle analysis of HCT-116 cells after 18 hours exposure to 100 $\mu\text{g/ml}$ of enoxaparin and its oligosaccharides (2 and 7) in media containing 2% of FCS. Graph depicts quantification of the percentage of cells in G₁, S, and G₂ phases. Data represents the average of three individual samples per treatment of a typical experiment. Error bars depict standard deviations. * $p < 0.05$, ** $p < 0.01$ versus control.

2.4 Discussion

Human epithelial cell proliferation is stimulated by various growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [209]. However, the *in vitro* response of cells to growth factors including bFGF is cell specific [204,210]. A number of colonic epithelial cell lines established from human colon carcinoma, are currently used for *in vitro* investigations [194,204]. One of them used in this study is an aggressively growing but poorly differentiated as well as microsatellite unstable cell line called HCT-116 [204,211]. In line with studies showing that cell proliferation was dependent on FCS concentrations [212,213], the proliferation of HCT-116 increased concentration-dependently. On the other hand, we did not observe that HCT-116 cells significantly responded to bFGF stimulation, despite reports that described increased proliferation of HCT-116 cells in response to 0.1 nM bFGF by 14% and 19% [57,204]. The differences in experiment setting such as initial cell number and the incubation of cells with different serum concentrations (0 or 5%) could explain the discordant observations. Nonetheless, HCT-116 cell line has been described as a bFGF-independent and EGF-independent colon cancer cell line [194,214].

Considerable interest has focussed on a potential role of heparins in accelerating bFGF-stimulated proliferation to assist wound healing [57,64]. In the context of the study, the binding of bFGF to its high affinity receptors was reported to be facilitated by HS or heparin [215]. It is thought that heparins can act in parallel with bFGF to stimulate colon epithelial cell proliferation [57,204]. Heparins were also shown to affect the signalling of bFGF in a concentration-dependent fashion. While UFH at low concentrations (0.1 - 30 nM) enhanced bFGF receptor binding and thus stimulated cell growth, inhibition of bFGF binding to its receptor by UFH was demonstrated at higher concentrations (10 μ M) [216]. In our

study, the possible growth effects of heparins and bFGF were investigated in media with reduced fetal calf serum concentration but not in other commercially available improved serum-reduced media such as Opti-minimal essential medium (MEM). So far, it is unclear if the response of HCT-116 epithelial cells to heparins or bFGF would be different in other media formulations such as OPTI-MEM. On the other hand, a previous study demonstrated only a maximum growth increase of 14% in response to 0.1 nM bFGF in serum-free medium [204], thus we did not pursue the growth effect of bFGF on HCT-116 epithelial cells in serum-free media and further. In view of the uncertainty in the literature and no apparent effect of bFGF on HCT-116 cell proliferation in our study, we therefore focused on the sole influence of heparins on colonic epithelial cell proliferation.

Together with previous reports [46,177,217], the data presented here demonstrate that in addition to their anticoagulation activity, heparins can effectively modulate additional biological functions. This study concentrated on the growth inhibitory activity of UFH and LMWHs. We and others demonstrated that under cell culture conditions of reduced serum concentrations, UFH and LMWHs can dose-dependently inhibit proliferation of different cell lines *in vitro* [188,218]. It has to be noted that in contrast to our experiments that are characterized by reduced serum levels, some investigators reported a reduced cell proliferation by UFH in the presence of 10% or even 20% v/v FCS [178,182,219]. However, in these studies in order to increase the sensitivity of cells to the anti-proliferative effect of UFH, cells were arrested by serum-starvation for up to 96 hours before the exposure to heparins, which effectively tests the ability of UFH to delay the re-entry of synchronized cells into the cell cycle. This approach is quite different to our experimental setting, which measures the inhibition of cell proliferation in unsynchronized cells.

Although the majority of studies described a significant inhibition of cellular proliferation by heparins, some reports also indicated that increased cell death might be associated with the observed effects in some cell lines [185,188,190]. In the experimental setting described here, increased cell death could have well accounted for the reduced colony formation in the presence of heparins. However, using three different assays no significant levels of either necrosis or apoptosis could be detected in the cell lines tested with both UFH and LMWHs. This is in line with other studies [205,206], in which heparins do not affect cell viability or the apoptotic levels. At the same time, it cannot be completely excluded that some cells might respond to exposure to heparins with cell death in different experimental settings such as extended exposure to high heparin concentrations. Moreover, compared to cancer cell lines used in this study, primary cells such as endothelial cells [207] and mesenchymal stromal cells [185] have been reported to be more susceptible to heparin-induced cell death.

The current literature strongly suggests that the cell cycle effects of heparins we and others observed are not cell type specific, since inhibition of proliferation was demonstrated in cells from a multitude of tissues and organisms [141,182,184,190,220]. In this context, it is important to note that some studies indicate that different cell types might have slightly different dose-effect-responses to UFH [178]. Overall, a wide range of UFH and LMWHs concentrations have been investigated and, dependent on the types of heparins and cell culture conditions used, effective concentrations varied only slightly. The inhibitory effect of heparins on cell proliferation was reported to be concentration-dependent [220,221]. However, in our experimental setting, there was a lack of true dose-dependent effect of heparins on cell proliferation which could be due to the specific range of heparin concentrations tested, a potential lack of sensitivity of the assay used or the use of different cell lines. Nevertheless, most studies agree that cellular proliferation is significantly

inhibited by UFH at a dose between 10 µg/ml and 100 µg/ml [178,179,181,182,218]. For enoxaparin, 25-50 µg/ml were reported to significantly inhibit proliferation of epithelial cells [188,205], while the minimum inhibitory concentration of dalteparin was found at 10 IU/ml (approximately 64 µg/ml) [190]. Our results describe reduced proliferation between 10 µg/ml and 400 µg/ml, with a significant reduction at 10 µg/ml of enoxaparin and 100 µg/ml for dalteparin or UFH, respectively, and therefore largely mirror the effective dose ranges described in previous studies. Thus, the effectiveness of these compounds might be less dependent on the cell type but rather more dependent on the experimental setting and more importantly on the origin of the compound.

Despite the well-established, standardized and quality-controlled methods of heparin production, heparins (UFH and LMWHs) remain complex mixtures of heterogeneous oligosaccharides and we have shown here that these oligosaccharides contain different and interestingly even opposing biological activities. This observation for the first time rationalizes the variation in the responses reported by different laboratories when using heparins. Heparins are available from different sources and manufacturers [222], and contain different anti-proliferative properties [223]. Although all commercially available heparins are characterized by their specific anticoagulant activity based on anti-factor Xa and thrombin activity, this does not guarantee a uniform composition of other activities, since they are currently not tested for. Thus, differences in composition other than the overall anticoagulant activity in preparations from different manufacturers and sources are likely to contribute to the differences seen when testing the non-anticoagulant activities. In support of this hypothesis, it was suggested previously that the compositional differences in non-anticoagulant oligosaccharides between batches of enoxaparin could be responsible for the inconsistent patient response to treatment of an inflammatory skin condition [129,138]. Furthermore our results can for the first time explain the conflicting literature with regards

to the pro- and anti-proliferative effects that have been described for heparins [194,195,224]. Based on our observation that enoxaparin consists of pro- and anti-proliferative oligosaccharides, it can be hypothesized that only small variations in the quantity and presence of specific oligosaccharides can alter the overall proliferative response to enoxaparin and also likely other heparins. This question is currently under investigation by other student in our laboratory.

Repeating disaccharides of an alternating sequence of uronic acid and glucosamine serve as the basic building blocks of heparin chains. However, sulphation groups can be unevenly distributed along the chains and serve as a means to introduce significant biological complexity [45]. Depending on the sources of raw material and the conditions of the purification process, unique structural features of the final products can be found from the extract [49,208]. This may include the presence of more, less or inactive moieties, or uncharacterized heparin molecules from the natural occurrence of GAGs in the extracted tissues [225]. For example, in 2007 and 2008 hundreds of hypersensitive reactions were reported worldwide after administration of heparin preparations contaminated with an impurity known as oversulphated chondroitin sulphate [136]. It is believed that this contaminant was introduced to certain batches of heparin during the extraction and manufacturing process [226]. This highlights the compositional complexity and structural variability that is currently present in the commercially available heparin mixtures, which is likely to represent a significant potential for varying clinical responses.

In order to identify oligosaccharides that are responsible for altered cellular proliferation in this study, enoxaparin was fractionated using IC, which generates molecules of varying size and sulphation status with and without anticoagulant activity [76]. This study demonstrates that not all oligosaccharides result in significant changes to the mitogenic

activity of cells. The selection of enoxaparin oligosaccharides for comparison depends not only on the differential effects on proliferation but also their inherited anticoagulant activity [76]. The antithrombin binding of enoxaparin is due to the presence of pentasaccharide sequence on the enoxaparin chains. Oligosaccharide 2 was selected because it contained the highest anti-proliferative activity ($-76.6 \pm 13.1\%$) and was the smallest molecule (disaccharide) from the mixtures of oligosaccharides. The disaccharide is distinctively shorter than the required smallest sequence of pentasaccharide for antithrombin binding [44], and thus is very unlikely to have any anticoagulant activity. On the other hand, oligosaccharide 7 represents hexasaccharides with only low anticoagulant activity [76] that can significantly increase the mitogenic activity of cells ($61.6 \pm 9.3\%$). This suggests that specific structures of heparin oligosaccharides are responsible for their effects on proliferation.

On another point, although oligosaccharide 11 resulted in $42.6 \pm 11.9\%$ inhibition of colony formation, oligosaccharide 11 represents decasaccharides (dp10) and has relatively higher anti-factor Xa activity compared to oligosaccharides 2 and 7 [134]. Similarly, oligosaccharide 12 (dodecasaccharide) which had the highest pro-proliferative activity was not selected for further tests since it displayed the strongest anticoagulant activity [134]. This is not surprising since it was previously reported that dodecasaccharides display significantly increased anti-coagulant activity as they contain two antithrombin binding pentasaccharides [227]. In contrast to our data, dodecasaccharide or even larger fragments have been reported to possess anti-proliferative activity *in vitro*, while hexasaccharides were previously identified as the minimum chain length for any anti-proliferative activity [141,221], while in contrast to our data disaccharides were only associated with very low anti-proliferative effects [141]. At the same time, Garg and colleagues concluded that size *per se* does not determine the anti-proliferative activity of heparin oligosaccharides [217]. This discrepancy

is again likely caused the different methods of oligosaccharide preparation from UFH, which are predominantly based on different chemical or enzymatic reactions. It is noteworthy that chemical or enzymatic digestion significantly increase the structural heterogeneity of heparins, causing variations in chain length and the substitution of important chemical groups present on glucosamine and urinate residues [49,208]. Therefore, even subtle changes in preparation could conceivably lead to different compositions and pharmacological profiles of heparins and thus different experimental results. Overall, the data by us and others illustrate that comparative studies from different labs using heparins from different sources and preparative methods are unlikely to lead to consistent results unless the detailed structure of individual oligosaccharides can be identified.

Even though a large body of evidence shows that heparins can influence cell growth, the details on how heparins regulate or modulate cell proliferation are still lacking. While further studies are needed to uncover the detailed mechanism by which heparins affect cellular proliferation, this study provides the framework for the standardized isolation of enoxaparin oligosaccharides with pro- and anti-proliferative activities. Furthermore, this standardized isolation of oligosaccharides is a crucial pre-requisite for exploring other potential therapeutic uses of heparin oligosaccharides.

2.5 Conclusion

Using a powerful chromatographic technique, this study has for very first time demonstrated that different oligosaccharides obtained from intact heparin have different and even opposing effects. Out of all oligosaccharides tested, oligosaccharide 2 (disaccharide) showed the largest anti-proliferative effect while oligosaccharide 7 (hexasaccharide) profoundly stimulated proliferation. Since oligosaccharide 2 is too small to have any anticoagulant activity and oligosaccharide 7 has only minimal anticoagulant activity, the effects of

heparins on cellular proliferation appear largely independent of their anticoagulant activity. All commercially available heparins have comparable anticoagulant activities; however, their non-anticoagulant oligosaccharide content greatly differs among the different heparin preparations. The composition of non-anticoagulant oligosaccharides generally depends on the source of isolation and the type of preparation employed. Therefore, depending on the structural characteristics of non-anticoagulant oligosaccharides, heparins can have either pro- or anti-proliferative effects. This study provides the platform for future studies determining the non-anticoagulant characteristics of oligosaccharides responsible for the different cellular effects as well as investigating their biological properties *in vivo*.

Chapter 3

Chapter 3 Orally Administered Enoxaparin Ameliorates Acute Colitis by Reducing Macrophage-associated Inflammatory Responses

3 Abstract

Inflammatory bowel diseases, such as ulcerative colitis, cause significant morbidity and decreased quality of life. The currently available treatments are not effective in all patients, can be expensive and have potential to cause severe side effects. This prompts the need for new treatment modalities. Enoxaparin, a widely used antithrombotic agent, is reported to possess anti-inflammatory properties and therefore we evaluated its therapeutic potential in a mouse model of colitis. Acute colitis was induced in male C57BL/6 mice by administration of dextran sulphate sodium (DSS). Mice were treated once daily with enoxaparin via oral or intraperitoneal administration and monitored for colitis activities. On termination (day 8), colons were collected for macroscopic evaluation and cytokine measurement, and processed for histology and immunohistochemistry. Oral but not intraperitoneal administration of enoxaparin significantly ameliorated DSS-induced colitis. Oral enoxaparin-treated mice retained their body weight and displayed less diarrhoea and faecal blood loss compared to the untreated colitis group. Colon weight in enoxaparin-treated mice was significantly lower, indicating reduced inflammation and oedema. Histological examination of untreated colitis mice showed a massive loss of crypt architecture and goblet cells, infiltration of immune cells and the presence of oedema, while all aspects of this pathology were alleviated by oral enoxaparin. Reduced number of macrophages in the colon of oral enoxaparin-treated mice was accompanied by decreased levels of pro-inflammatory cytokines. Oral enoxaparin significantly reduces the inflammatory pathology associated with DSS-induced colitis in mice and could therefore represent a novel therapeutic option for the management of ulcerative colitis.

3.1 Introduction

Ulcerative colitis (UC) is a chronic inflammatory condition with millions of sufferers worldwide [228]. In UC, chronic inflammation of the inner lining of the colon leads to abdominal pain, diarrhoea, bloody stool and weight loss, and results in decreased quality of life. Currently, pharmacological and surgical interventions are the two main management approaches for UC [2]. Drugs such as corticosteroids, aminosalicylates, and immunosuppressants, which aim to decrease inflammation, show limited effectiveness for long term remission and are associated with significant side effects [36]. Monoclonal antibodies, such as infliximab, that inhibit tumour necrosis factor (TNF- α) have shown considerable success [229]. However, they are expensive, about 30 - 40% of patients do not respond to TNF- α inhibitors and in up to 40% of patients the initial therapeutic response is lost after 1 - 2 years [39]. Surgery is reserved for about 20 - 30% of patients who are unresponsive to medication and develop life-threatening complications such as perforation, refractory rectal bleeding, toxic megacolon and fulminant colitis [2]. Even after surgery, patients are predisposed to the risk of complications such as small bowel obstruction, anastomotic strictures, pouchitis and pouch failure [36].

Therefore, the search for safer and more effective agents for the management of UC continues. Among these agents, heparins (unfractionated heparin, UFH and low-molecular-weight heparins, LMWHs) have attracted much interest for their paradoxical responses. In clinical practice, heparins are widely used for the treatment and prophylaxis of venous thromboembolism [48]. However, many studies have demonstrated therapeutic usefulness of heparins apart from their anticoagulant activity, of which their anti-inflammatory properties have attracted much interest among researchers around the world [46].

Heparins are GAGs and are composed of a heterogeneous mixture of highly sulphated anticoagulant and non-anticoagulant molecules known as oligosaccharides [49]. LMWHs have replaced UFH clinically to a large extent because of their improved pharmacokinetic properties and fewer side effects. Thromboembolic complications have been reported in patients with UC and it was found serendipitously that UFH is linked to remission of UC when patients were treated with UFH for deep vein thrombosis or pulmonary embolism [43]. Since then, several pre-clinical as well as open-labelled clinical studies have demonstrated the usefulness of heparins in UC [73,109,114,119].

However, some pre-clinical and clinical studies have shown little or no significant clinical advantage of LMWHs compared to placebo when used for the management of UC [115-117,230,231]. The observed differences in clinical outcome could be due to the fact that these studies were not only heterogeneous in regards to their methodological approaches and definitions for clinical outcomes, but also used different heparins. For example, two LMWHs, dalteparin and nadroparin, have reported to be effective in patients with steroid resistant UC [112,113]. On the other hand, tinzaparin (another LMWH) has shown no additive benefit over standard therapy in patients with UC [117]. LMWHs are prepared by different chemical or enzymatic processes and are dissimilar to each other in both their physical and chemical properties [49]. Many studies have shown real and significant differences between various LMWHs based on their oligosaccharide analysis [133,232]. Dotan *et al.* found that enoxaparin may be an effective therapy for active UC and may delay or preclude the need for corticosteroid treatment [114]. On the other hand, Zazos and co-workers reported no additional therapeutic benefit of enoxaparin compared with classical therapy. *In vivo* investigations of enoxaparin in other inflammatory conditions (e.g. lichen planus) have also reported contradictory results [129,138]. Commercially available enoxaparin or any other LMWH is normally standardized according to its anticoagulant

activity (anticoagulant oligosaccharides) but not the other molecules (non-anticoagulant oligosaccharides), which results in batch-to-batch variation [132,140]. It has been reported that the differences in non-anticoagulant oligosaccharides between different batches of enoxaparin may be responsible for the inconsistent clinical findings [138]. We have also previously reported the structural variations in two different batches of enoxaparin formulated by the same manufacturer [140].

It is known that the absorption of LMWHs from the gut is poor after oral administration because of their hydrophilicity, high negative charge and large size [233]. Therefore, in previous clinical studies LMWHs were administered parenterally. However, such macromolecules are composed of anticoagulant and non-anticoagulant oligosaccharides and hence the risk of bleeding is increased when used for medical conditions other than where an anticoagulant effect is required. For example, in one study, administration of a LMWH resulted in the massive haemorrhage in a patient with UC [234]. Therefore, studies investigating the role of LMWHs in inflammatory conditions have used low parenteral doses in an attempt to avoid bleeding complications. However, it has been reported that their anti-inflammatory activities are expressed with high LMWH concentrations where the anticoagulant effects also predominate [120]. If heparins are administered orally, because of their lack of systemic absorption, they can be delivered to the site of inflammation in UC. This route would potentially minimize the risk of bleeding as well as allow the use of high doses. Given the mixed findings with the use of enoxaparin in UC, we investigated and compared the efficacy of orally and parenterally administered enoxaparin utilizing the most widely used animal model of chemically-induced colitis, with the aim of determining the probable underlying mechanisms of enoxaparin in attenuating colitis.

3.2 Materials and methods

3.2.1 Animal colitis model

All animal experiments were approved by the Animal Ethics Committee of the University of Tasmania (Ethics approval number: A13576) and conducted in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (8th Edition 2013). Male C57BL/6 mice, (aged 8 - 10 weeks; 21 - 30 g, average \approx 25 g), were obtained from the University of Tasmania animal breeding facility and housed in a temperature-controlled environment with a 12-hour day/ night light cycle. Individual body weights were assessed daily over an initial acclimation period of 7 days. All mice were non-fasting and had access to food and autoclaved tap water for drinking *ad libitum* during experiment. Colitis was induced by feeding mice with 3% w/v dextran sulphate sodium (DSS, MW = 40000-50000, USB, Affymetrix Inc, Ohio, USA) dissolved in drinking water from day 1 to day 8. Control mice received water without DSS from day 1 to day 8.

3.2.2 Formulation of enoxaparin

Enoxaparin solution for intraperitoneal administration was prepared by diluting enoxaparin (100 mg/ml; Clexane, Sanofi Aventis, NSW, Australia) with water for injection to obtain a final concentration of 0.25 mg/ml. Food mash containing enoxaparin for oral administration was prepared by dissolving 9.6 g sucrose (4% w/w of food mash) in 100 ml autoclaved tap water followed by addition of 0.4 ml of enoxaparin (100 mg/ml) solution. Powdered food (130 g) (Barastoc Rat & Mouse Pellet, Ridley AgriProducts, Melbourne, Australia) was then added slowly with constant stirring to this solution to prepare a homogenous food mash mixture. Then 3 g of this mixture containing 0.5 mg of enoxaparin was transferred to food trays. Each food tray was stored at -20 °C until used.

3.2.3 Treatment with enoxaparin

Mice were assigned randomly into six groups: i) untreated healthy control mice (C, n = 6) ii) healthy mice treated with oral enoxaparin (C+OE, n = 3) iii) healthy mice treated with intraperitoneal enoxaparin (C+IPE, n=3) iv) mice which received DSS (through drinking water) and vehicle (water for injection or food mash) (DSS, n = 14) v) mice which received DSS in drinking water and treated with oral enoxaparin (DSS+OE, n = 12) and vi) mice which received DSS in drinking water and were treated with intraperitoneal enoxaparin (DSS+IPE, n = 6). Enoxaparin (20 mg/kg/day for oral or 0.5 mg/kg/day for intraperitoneal injection) was given from day 1 to day 7.

3.2.4 Evaluation of intestinal inflammation

Mice were monitored daily for change in body weight. Determination of body weight daily is crucial to monitor the development of colitis and its severity. Stool consistency and the presence of blood in the stool were scored daily (Table 3.1) [235]. The anal area was examined for the presence of blood and faeces was collected and tested for the presence of blood using Hemocult II slides (Beckman Coulter Inc., California, USA).

Table 3.1 Scoring system for stool consistency and blood in stool [235]

Score	Stool consistency	Blood in faeces
0	Normal formed pellet	None/ negative
1	Soft but still formed (pasty when pressed)	Hemocult +
2	Loose stool	Visual blood in stool
3	Watery stool/ diarrhoea	Gross bleeding/ bloody anus

3.2.5 Termination of experiment and tissues sampling

Mice were killed by carbon dioxide inhalation followed by cervical dislocation on day 8. The entire colon was carefully removed and examined macroscopically. The colon length was measured before opening it longitudinally for observation of colonic content. After mechanical cleaning, colon weight was determined and the colon tissue was then divided longitudinally for histology and measurement of cytokine levels.

3.2.6 Histologic evaluation of colitis

Colon tissue was fixed with 10% v/v buffered formalin and processed for paraffin embedding. Paraffin embedded tissue was cut into sections (4 μ m thickness) before using for hematoxylin and eosin (H&E) staining and immunohistochemistry. All H&E sections were graded blindly for the severity of tissue damage at distal and proximal regions as described previously [236-238].

3.2.7 Immunohistochemistry

For immunohistochemical staining, antigen retrieval was performed by incubating the sections for 10 minutes at 97 °C in 1 mM EDTA buffer, pH 8 or 10 mM citrate buffer, pH 6. Activity of endogenous peroxidase was blocked by incubating sections with 3% v/v hydrogen peroxide for 20 minutes. Sections were then washed with 0.05 M Tris-buffered saline containing 0.5% v/v Tween 20 (TBST), pH 7.6. Subsequently, sections were incubated with serum-free protein block (Dako, Victoria, Australia) for 10 minutes. Colon sections were then incubated with primary antibodies: anti-F4/80 (ab111101, Abcam, Cambridge, UK, 1:100); anti-claudin-4 (ab15104, Abcam, 1:200); anti-occludin (ab64482, Abcam, 1:50) or its isotype control antibody (monoclonal rabbit immunoglobulin G, ab172730, Abcam, assay dependent concentration) overnight at 4 °C or at room temperature

for 1 hour. Sections were then washed 3×5 minutes and allowed to react with secondary antibody: anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (ab7090, Abcam, 1:300) at room temperature for 1 hour. Histological signal was developed using 3,3'-diaminobenzidine in a chromogen solution (ab64238, Abcam, 1:50) before counterstaining the sections with hematoxylin. Sections were dehydrated and mounted using mounting medium (Dako, Victoria, Australia) and then examined microscopically (Leica DM2500, Image Pro Plus 7.0 software) for positively stained cells. The number of F4/80-positive macrophages was counted at high-power field ($400 \times$ magnification) and averaged from ten fields.

For double immunofluorescence staining, sections were dewaxed and rehydrated before antigen retrieval using 10 mM citrate buffer, pH 6 for 15 minutes at 97°C . Sections were incubated with serum-free protein block (Dako, Victoria, Australia) and permeabilized with 0.4% v/v Triton-X at room temperature for 30 minutes. Sections were incubated with primary antibodies: anti-F4/80 (ab16911, Abcam, 1:25); anti-IL1 β (ab9722, Abcam, 1:300), anti-iNOS (ab136918, Abcam, 1:100), anti-mannose receptor (CD206) (ab64693, Abcam, 1:2000) overnight at 4°C or at room temperature for 1 hour. The omission of primary antibodies and replacement with isotype controls (rabbit immunoglobulin G and normal rat serum at assay-dependent concentration) served as independent negative controls. Sections were washed with TBST 3×10 minutes and incubated with species-specific secondary antibodies: anti-rat IgG H&L AlexaFluor 594 (ab150160, Abcam, 1:1000) and anti-rabbit IgG H&L AlexaFluor 488 (A11070, Thermo Fisher Scientific, Melbourne, Australia, 1:1000) at room temperature for 2 hours. Sections were rinsed 3×10 minutes, followed by a quick wash with distilled water before mounting using Glycerol Mounting Medium (Abcam) that contained 4',6-diamidino-2-phenylindole (DAPI) and 1,4-diazobicyclo-2,2,2-octane (DABCO). Labelled tissues were visualized using a Leica DM LB2 microscope.

Fluorescence images ($400 \times$ magnification) were captured using NIS-Elements 4.13 (Nikon) software. The number of fluorescence-positive cells was counted from five representative high-power fields ($400 \times$ magnification) per tissue section and expressed as percent of double positive cells/ total number of macrophages.

3.2.8 Tissue explant culture and measurement of cytokine levels

Each collected tissue was cut, weighed and washed with cold PBS before transferring to a 12 well plate containing 1 ml/ well of RPMI 1640 culture medium (In Vitro Technologies Pty Ltd, Melbourne, Australia) supplemented with 10% v/v foetal calf serum (Gibco, Life Technologies, Melbourne, Australia), penicillin (100 mU/L) and streptomycin (100 mg/L) (Sigma-Aldrich Pty Ltd, Sydney, Australia). After 24 hours of incubation, supernatant was collected from each well and stored at -80°C until further analysis. The cytokine levels were determined by immunoassay using a Bio-Plex Pro Mouse cytokine 23-plex kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following manufacturer instructions. Briefly, standards were prepared by reconstituting the cytokine standard with culture media. Solution containing coupled magnetic beads was added into each well of a 96 well plate. Standard and samples were then added in duplicate and incubated for 30 minutes. After that, detection antibodies were added and incubated before final incubation with streptavidin-phycoerythrin. Cytokine levels were measured and analysed using a Bio-Plex 200 instrument (Bio-Rad Laboratories) and Bioplex Manager software, version 6 (Bio-Rad Laboratories) respectively. The cytokine levels were normalized by dividing the cytokine results (pg/ml) by the measured biopsy weight (mg) to obtain pg/ml of cytokines/ 10 mg of tissue.

3.2.9 Statistical analysis

Results are presented as mean \pm standard deviation (SD), or points of minimum, median, mean, maximum and interquartile range. Using GraphPad Prism (version 6, GraphPad Software Inc, CA, USA), statistical significance was evaluated using one or two way analysis of variance (ANOVA) followed by multiple comparison test: Dunnett's test to evaluate the difference between each treatment group and the colitis control group or Tukey's test to determine the differences between different groups. Pearson's correlation coefficient (r^2) was determined for the relationship between two variables when necessary. A p value of < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Orally administered enoxaparin ameliorates colitis-induced weight loss

In line with previous reports [239], we observed a reduction of approximately 15% body weight over an 8 day DSS treatment period (Fig. 3.1A). Oral enoxaparin attenuated body weight loss where the difference in the body weight loss between DSS-treated (DSS) and oral enoxaparin treated (DSS+OE) mice was significant on day 6 (2.9%, $p = 0.0007$), day 7 (4.9%, $p < 0.0001$) and day 8 (8.1%, $p < 0.0001$) (Fig. 3.1A). On the other hand, difference in the body weight loss between DSS and intraperitoneal enoxaparin (DSS+IPE) was not significant from day 1 to day 8 (Fig. 3.1A).

3.3.2 Enoxaparin ameliorates colitis-associated diarrhoea and blood in stool

Over the treatment period, we observed an increased occurrence of loose stool and diarrhoea in mice receiving DSS (Fig. 3.1B). Consistent with an amelioration of weight loss, orally administered enoxaparin significantly reduced the occurrence of loose stool or diarrhoea on day 6 and day 7 (Fig. 3.1B). On the other hand, intraperitoneal enoxaparin also reduced the

mean stool consistency score on day 7 (Fig. 3.1B). Both oral and intraperitoneal enoxaparin significantly prevented the presence of blood in stool on day 5 only; this effect was not seen on day 6 and 7 (Fig. 3.1C). There was no statistical difference between oral and intraperitoneal enoxaparin treatment for both mean stool consistency and stool blood scores.

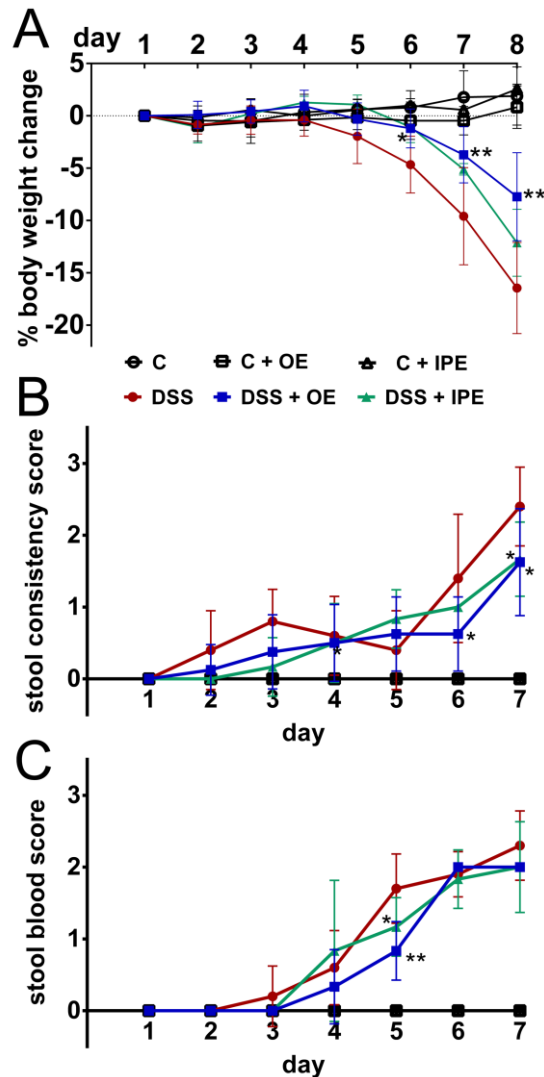


Figure 3.1 Effect of enoxaparin during acute colitis.

(A) Daily body weight changes during colitis induction in C57BL/6 mice with and without enoxaparin compared to healthy control. Stool samples collected from mice were scored for (B) consistency and the presence of (C) occult blood on a daily basis during experiment. Results are expressed as mean \pm SD of $n = 3 - 14$ mice. * $p < 0.05$ and ** $p < 0.01$. Control, C; control with oral enoxaparin, C+OE; control with intraperitoneal injection of enoxaparin, C+IPE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE; colitis with intraperitoneal injection of enoxaparin, DSS+IPE.

3.3.3 Orally administered enoxaparin alleviates macroscopic changes of the colon

Having observed beneficial clinical changes by orally administered enoxaparin, we then investigated the effects of enoxaparin in ameliorating macroscopic changes of colon (Fig. 3.2.1 and Fig. 3.2.2A). Acute colitis was associated with a significant shortening of the colon. The length of colon was reduced by 20.8% compared to healthy control (Fig. 3.2.2B). Oral enoxaparin significantly prevented shortening of colon by 10.2% ($p = 0.025$) compared to DSS. Intraperitoneal enoxaparin had no effect on the length of colon (Fig. 3.2.2B). Oral enoxaparin also reduced the severity of gross changes in the luminal contents of the distal colon compared to untreated colitis (Fig. 3.2.2C).

Colon weight change, a well-known independent marker of intestinal oedema and inflammation, was presented as colon weight over body weight. Oral enoxaparin suppressed the increase in relative colon weight by 14.2% ($p = 0.011$) compared to untreated colitis. On the other hand, intraperitoneal enoxaparin reduced the relative colon weight by only 4.2%, which was not significant ($p = 0.91$) (Fig. 3.2.2D).

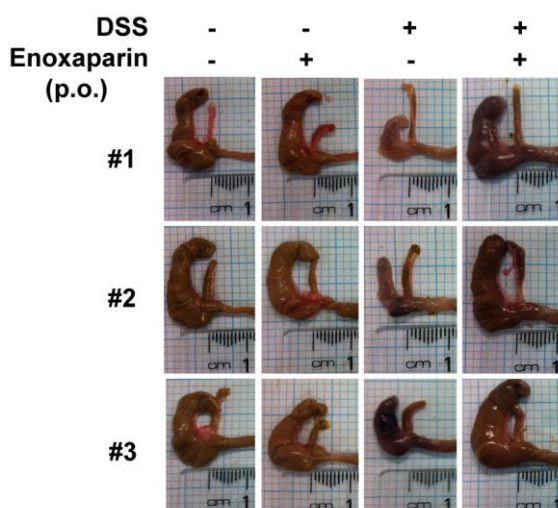


Fig. 3.2.1 Representative images of gross appearance of cecum.

C57BL/6 mice were given 3% w/v DSS in their drinking water from day 1 to day 8. Control mice were given water only. They were treated with or without oral (p.o.) enoxaparin. Cecums were collected on day of termination. Control, C; control with oral enoxaparin, C+OE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE.

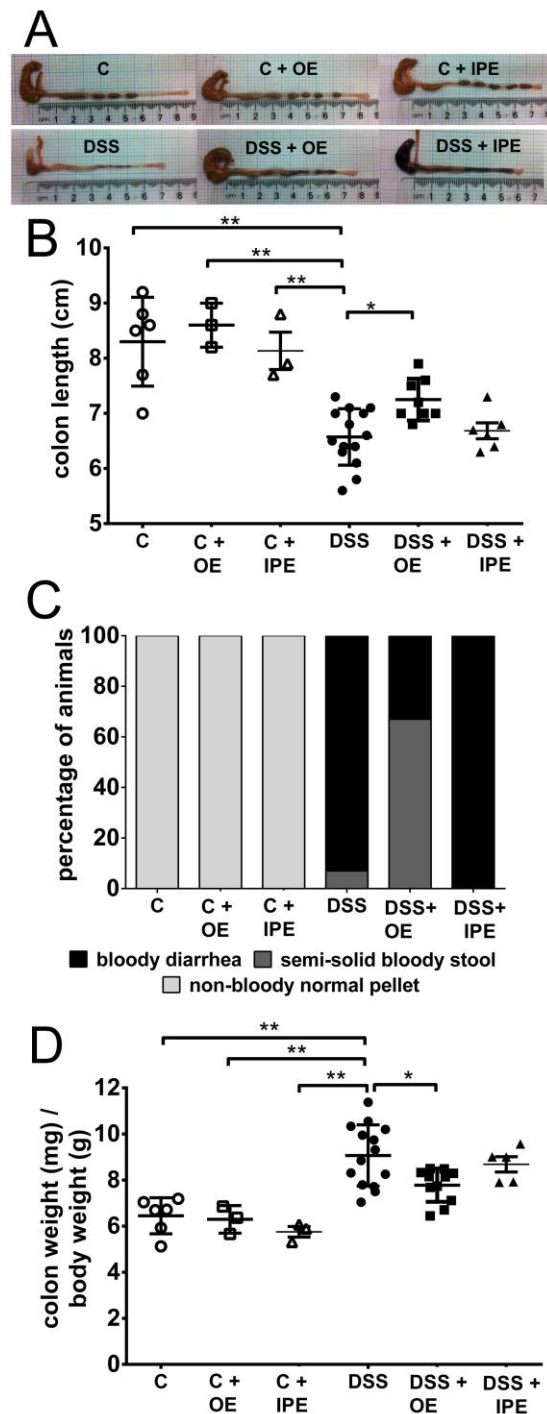


Figure 3.2.2 Effect of enoxaparin on macroscopic appearance of colon.

(A) Representative images of colons from mice treated with and without enoxaparin. (B) The colons were measured for their length. (C) The appearance of colon luminal content was evaluated before the measurement of colon weight. (D) The relative colon weight was presented as colon weight divided by body weight. Results are expressed as mean \pm SD of $n = 3 - 14$ mice. * $p < 0.05$ and ** $p < 0.01$. Control, C; control with oral enoxaparin, C+OE; control with intraperitoneal injection of enoxaparin, C+IPE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE; colitis with intraperitoneal injection of enoxaparin, DSS+IPE.

3.3.4 Orally administered enoxaparin decreases colon damage and infiltration of inflammatory cells

Colons of healthy untreated mice showed intact surface epithelium, intact mucosa and submucosa, non-disrupted crypt architecture, complete goblet cells with mucus vacuoles and only a small number of leukocytes (Fig. 3.3). In contrast, DSS treated mice showed structural damage and infiltration of inflammatory cells into the colon. Careful evaluation of colon tissues revealed regional DSS-induced injuries mainly confined to the distal colon where diffused destruction of crypt architecture, goblet cell loss, submucosa oedema and increased infiltration of inflammatory cells was evident (Fig. 3.3). Oral enoxaparin reduced the disruption of crypt architecture and epithelium, reduced goblet cell loss, showed less severe submucosa oedema, and also reduced infiltration of inflammatory cells (Fig. 3.3). The cumulative histological scores for both the proximal colon (Fig. 3.4A) and distal colon (Fig. 3.4B) were significantly lower in orally treated mice with colitis compared to untreated colitis (4 versus 8 and 16 versus 23 respectively). Intraperitoneal administration of enoxaparin did not reduce the colon damage and infiltration of inflammatory cells (Fig. 3.3). Also, the cumulative histological scores were not statistically different compared to DSS (Fig. 3.4).

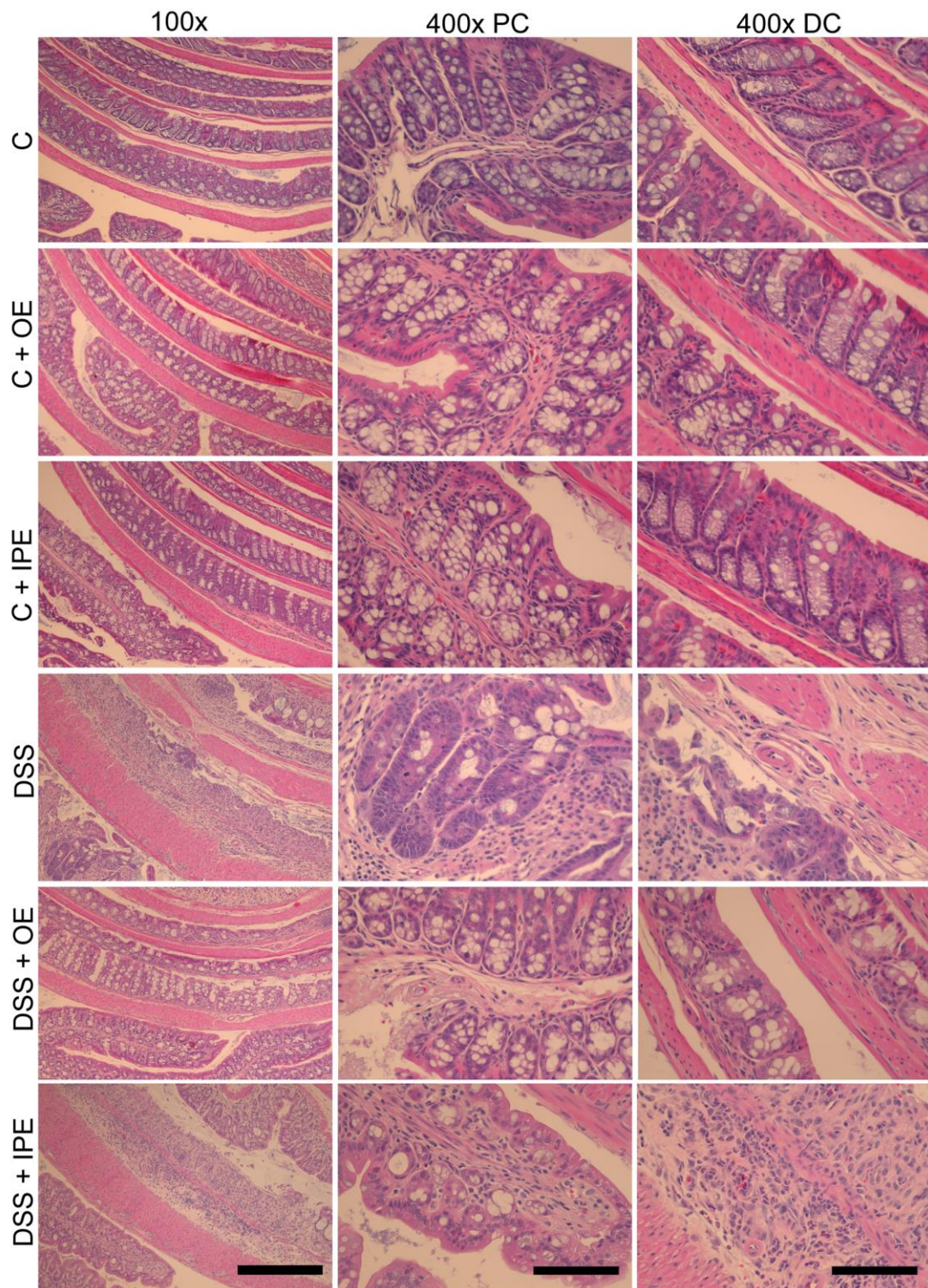


Figure 3.3 Effect of enoxaparin on histological changes of colon.

Representative hematoxylin and eosin stained colon sections of healthy and colitis mice with and without enoxaparin. Scale bars = 100 μ m for 400 \times and 400 μ m for 100 \times magnification. Control, C; control with oral enoxaparin, C+OE; control with intraperitoneal injection of enoxaparin, C+IPE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE; colitis with intraperitoneal injection of enoxaparin, DSS+IPE.

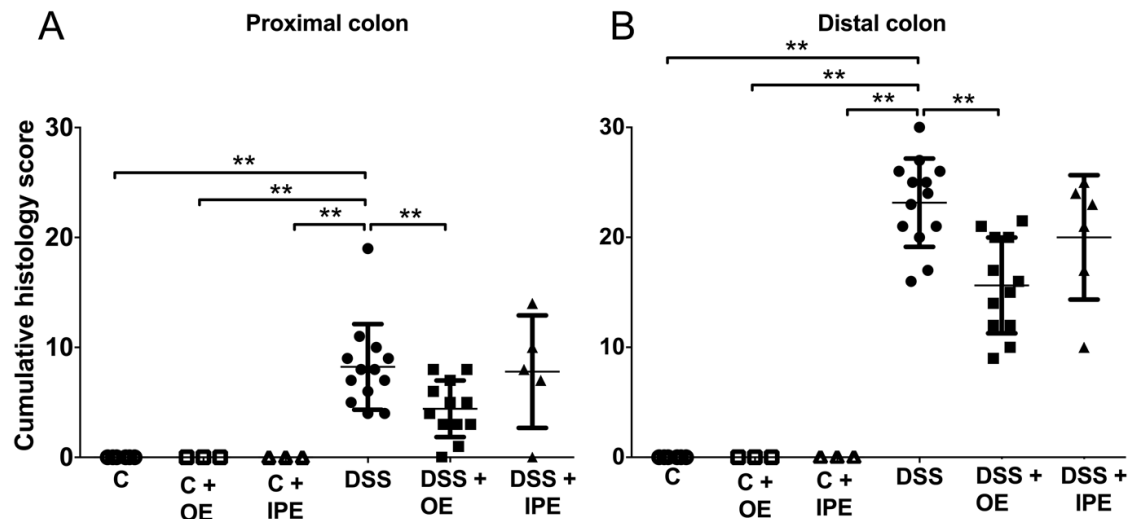


Figure 3.4 Effect of enoxaparin on histological changes of colon.

Cumulative histology damage scores for (A) proximal colon and (B) distal colon. Results are expressed as mean \pm SD of $n = 3 - 14$ mice. ** $p < 0.01$. Control, C; control with oral enoxaparin, C+OE; control with intraperitoneal injection of enoxaparin, C+IPE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE; colitis with intraperitoneal injection of enoxaparin, DSS+IPE.

3.3.5 Orally administered enoxaparin reduces the levels of pro-inflammatory cytokines

As expected, colon tissues of mice administered with DSS showed significant increase in a wide range of inflammatory cytokines (Fig. 3.5.1 and Fig. 3.5.2). Oral enoxaparin reduced the levels of a number of cytokines significantly. The levels of IL-1 α , IL-1 β , IL-10, MIP-1 α , MIP-1 β , G-CSF and GM-CSF were reduced by 44.6% ($p = 0.0002$), 40.4% ($p = 0.0011$), 54.5% ($p < 0.0001$), 63.6% ($p = 0.0002$), 62.3% ($p = 0.0263$), 51.6% ($p = 0.0013$) and 40.1% ($p < 0.0001$) respectively (Fig. 3.5.1 A-G). However intraperitoneal enoxaparin produced inconsistent response. For example, it reduced MIP-1 α level by approximately 45% (Fig. 3.5.1C). On the other hand, the levels of G-CSF, GM-CSF and IL-4 were increased significantly (Fig. 3.5.1 E, F, H). Unlike intraperitoneal enoxaparin, oral enoxaparin did not affect the levels of cytokines in healthy mice.

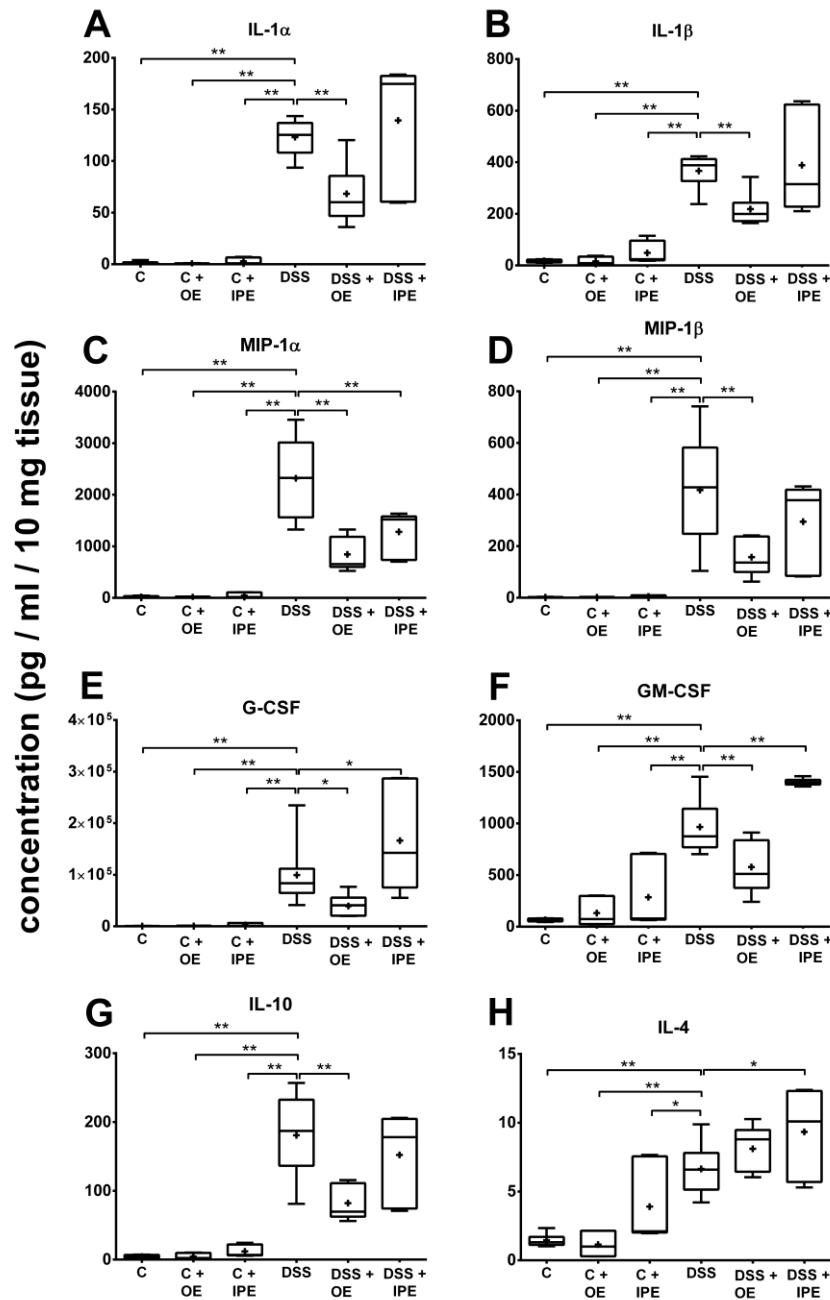


Figure 3.5.1 Effect of enoxaparin on colonic cytokine levels.

Distal colon tissues of mice were cultured for 24 hours. Supernatants were collected and cytokine levels were measured using Bio-Plex assay. Cytokine levels in the supernatant were normalized to tissue weight to obtain pg/ml of cytokines/ 10 mg of tissue. Results are expressed as minimum, 25th percentile, median, mean, 75th percentile and maximum of cytokine levels of 3 - 5 mice. * $p < 0.05$ and ** $p < 0.01$. Interleukin, IL; macrophage inflammatory protein, MIP; granulocyte colony-stimulating factor, G-CSF; granulocyte-macrophage colony-stimulating factor, GM-CSF; Control, C; control with oral enoxaparin, C+OE; control with intraperitoneal injection of enoxaparin, C+IPE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE; colitis with intraperitoneal injection of enoxaparin, DSS+IPE.

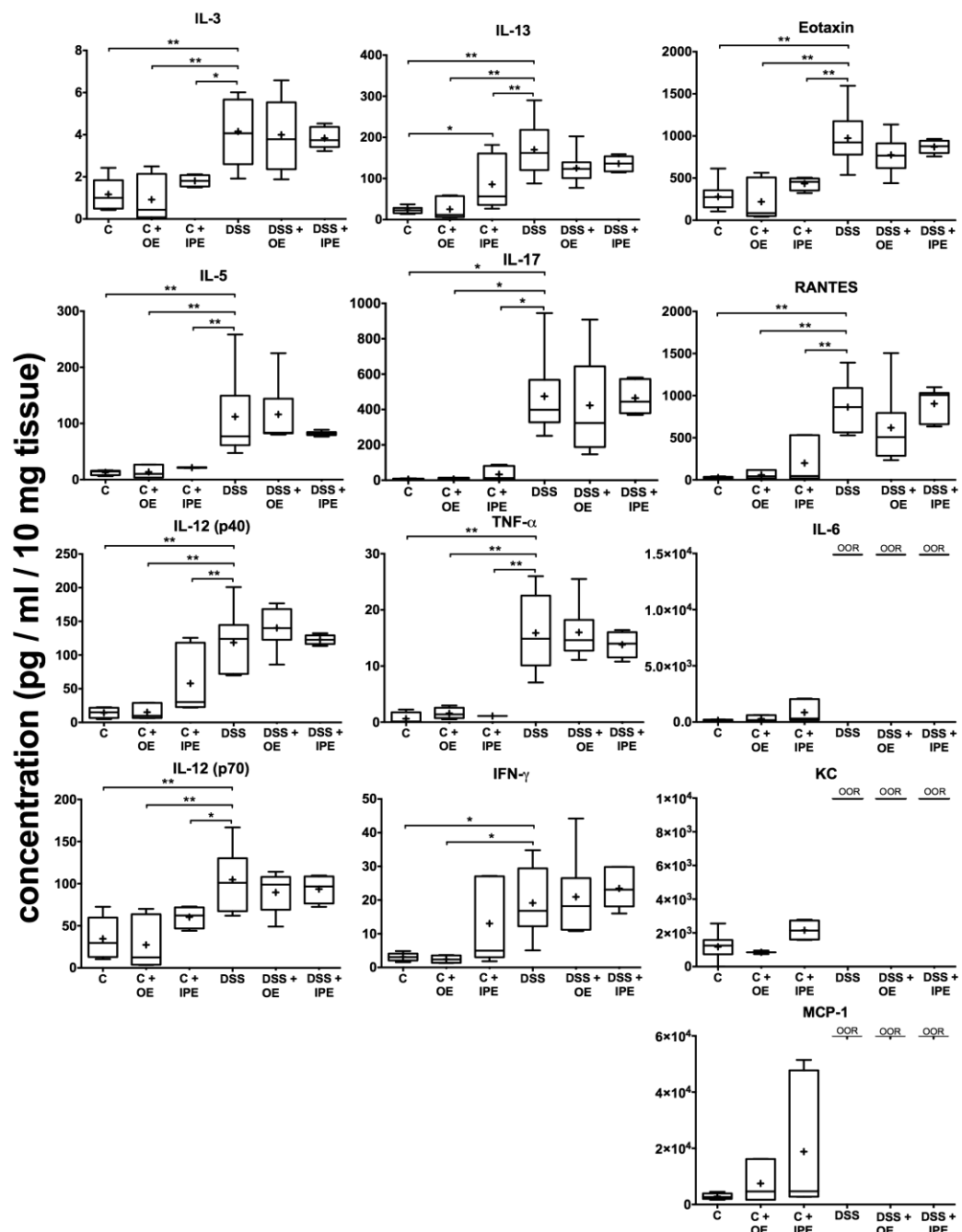


Figure 3.5.2 Effects of enoxaparin on colonic cytokine levels.

Distal colon tissues of mice were cultured for 24 hours. Supernatants were collected and measured for cytokine levels by using Bio-Plex assay. Cytokine levels in the supernatant were normalized to tissue weight to obtain pg/ml of cytokines/ 10 mg of tissue. Results are expressed as minimum, 25th percentile, median, mean, 75th percentile and maximum of cytokine levels of 3 - 5 mice. * $p < 0.05$ and ** $p < 0.01$. Out of range, OOR; Interleukin, IL; interferon, IFN; keratinocyte-derived chemokine, KC; monocyte chemotactic protein-1, MCP-1; regulated and normal T cells expressed and secreted, RANTES; tumor necrosis factor- α , TNF- α ; Control, C; control with oral enoxaparin, C+OE; control with intraperitoneal injection of enoxaparin, C+IPE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE; colitis with intraperitoneal injection of enoxaparin, DSS+IPE.

Since increased cytokine levels in the colonic mucosa of UC patients are closely related to the severity of inflammation and tissue damage [240-242], we determined the Pearson's correlation coefficients between cytokine levels (that statistically significantly decreased by oral treatment) and the percentage body weight change in individual mice on day 8. Consistent with clinical observations, cytokine levels significantly and positively correlated with the severity of body weight loss (Fig. 3.6 A-G). Mucosal concentrations of IL-1 α ($r^2 = 0.77$, $p < 0.0001$), IL-1 β ($r^2 = 0.70$, $p < 0.0001$), MIP-1 α ($r^2 = 0.69$, $p < 0.0001$), MIP-1 β ($r^2 = 0.58$, $p < 0.0001$), G-CSF ($r^2 = 0.61$, $p < 0.0001$), GM-CSF ($r^2 = 0.66$, $p < 0.0001$) and IL-10 ($r^2 = 0.74$, $p < 0.0001$) were each substantially correlated with the percentage of body weight loss.

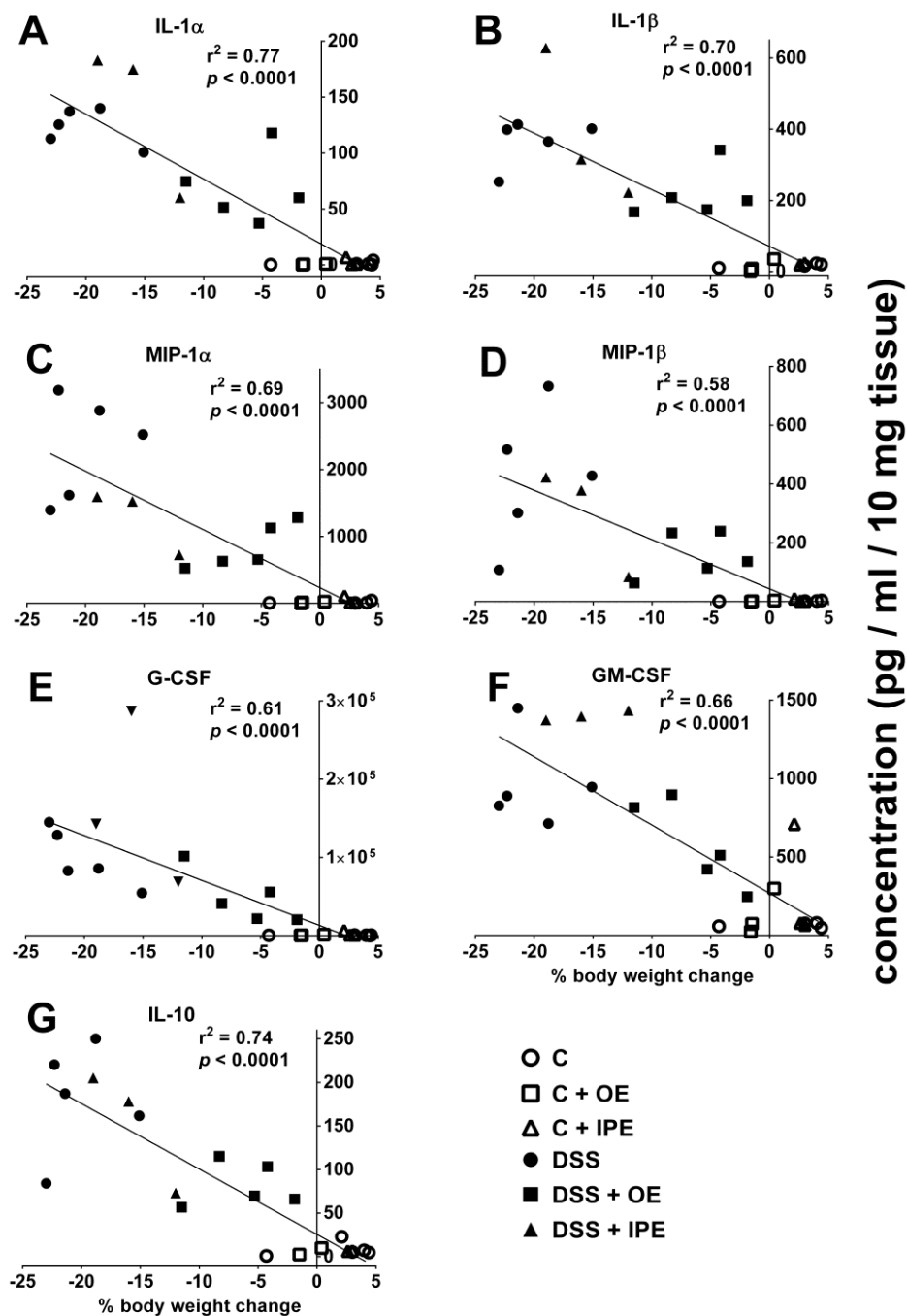


Figure 3.6 Correlation between colonic cytokine levels and body weight changes during acute colitis.

Changes in cytokine levels were correlated with the changes of body weight of individual mice. The value of the Pearson correlation coefficient (r^2) is reported and significance is indicated by p value. Interleukin, IL; macrophage inflammatory protein, MIP; granulocyte colony-stimulating factor, G-CSF; granulocyte-macrophage colony-stimulating factor, GM-CSF; Control, C; control with oral enoxaparin, C+OE; control with intraperitoneal injection of enoxaparin, C+IPE; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE; colitis with intraperitoneal injection of enoxaparin, DSS+IPE.

3.3.6 Orally administered enoxaparin affects macrophage numbers and differentiation status during colitis

Macrophages play an important role during acute colitis by secreting various pro-inflammatory mediators [243]. Therefore, we investigated if the observed reduction in cytokines could be a result of reduced number of macrophages in the colonic mucosa. When we compared the number of macrophages between different types of treatment, significantly higher macrophage numbers (33.3 ± 7.1 / field) were observed in the mucosa and submucosa in DSS-treated mice compared to healthy control mice (1.1 ± 1.2 / field) (Fig. 3.7). Consistent with the reduced cytokine levels, oral enoxaparin also significantly decreased the number of macrophages (17.8 ± 5.4 / field, $p < 0.0001$) in the mucosa and submucosa. On the other hand, intraperitoneal enoxaparin was not effective in decreasing the number of macrophages in the colon tissue (29.2 ± 6.5 / field) (Fig. 3.7).

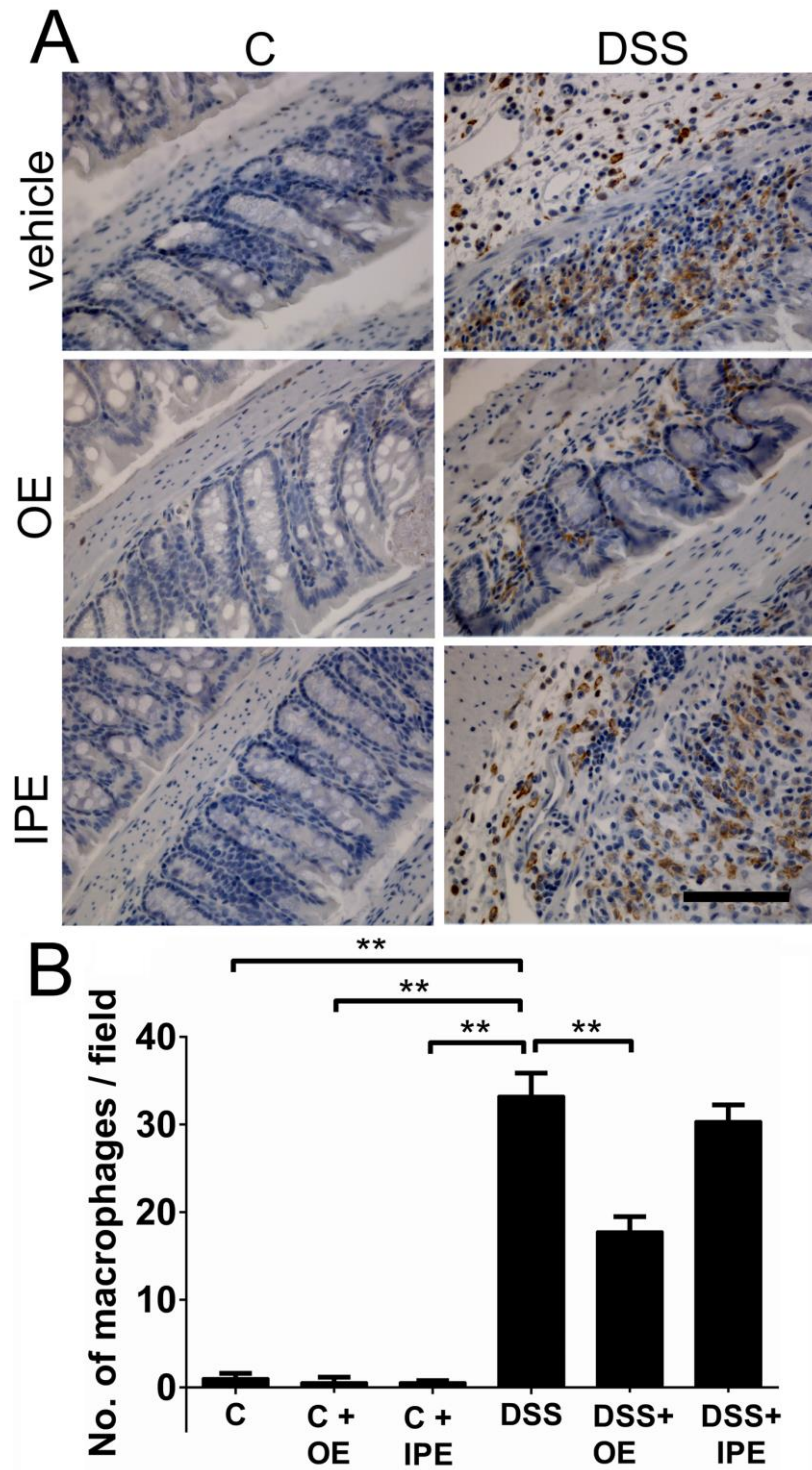


Figure 3.7 Effect of enoxaparin on macrophage infiltration into the inflamed colon.

(A) Representative immunostaining of F4/80-positive macrophages in the distal colon from healthy and colitic mice treated with and without enoxaparin. (B) Quantification of macrophages (F4/80⁺ cells) in the colons. Results are expressed as mean \pm SD of ten representative high-power fields per tissue section of 3 - 5 mice each. ** $p < 0.01$. Scale bar = 100 μ m for 400 \times magnification. Control, C; untreated colitis, DSS; oral enoxaparin, OE; intraperitoneal injection of enoxaparin, IPE.

In addition to the absolute numbers of macrophages, we also looked at their differentiation status. When we detected M1 macrophages (F4/80 and iNOS positive cells, Fig. 3.8A) in the colon tissue, only low numbers were observed mainly in the mucosa (double headed arrow) of healthy control colons. In response to DSS treatment, as expected large numbers of M1 macrophages were detected not only within the oedematous submucosa (arrow heads) but also within the mucosa (double headed arrow). In response to enoxaparin treatment, those macrophage numbers were significantly reduced with a few M1 cells present only in the mucosa (double headed arrow) (Fig.3.8A).

In contrast, when we looked at M2 macrophages (F4/80 and CD-206 positive cells, Fig. 3.8B), the majority of macrophages were present in the mucosa of healthy colon (double headed arrow). In response to DSS, only a very low numbers of M2 macrophages were present in the mucosa and submucosa, while in response to enoxaparin treatment, larger numbers of M2 macrophages were present in the mucosa and submucosa (Fig. 3.8B).

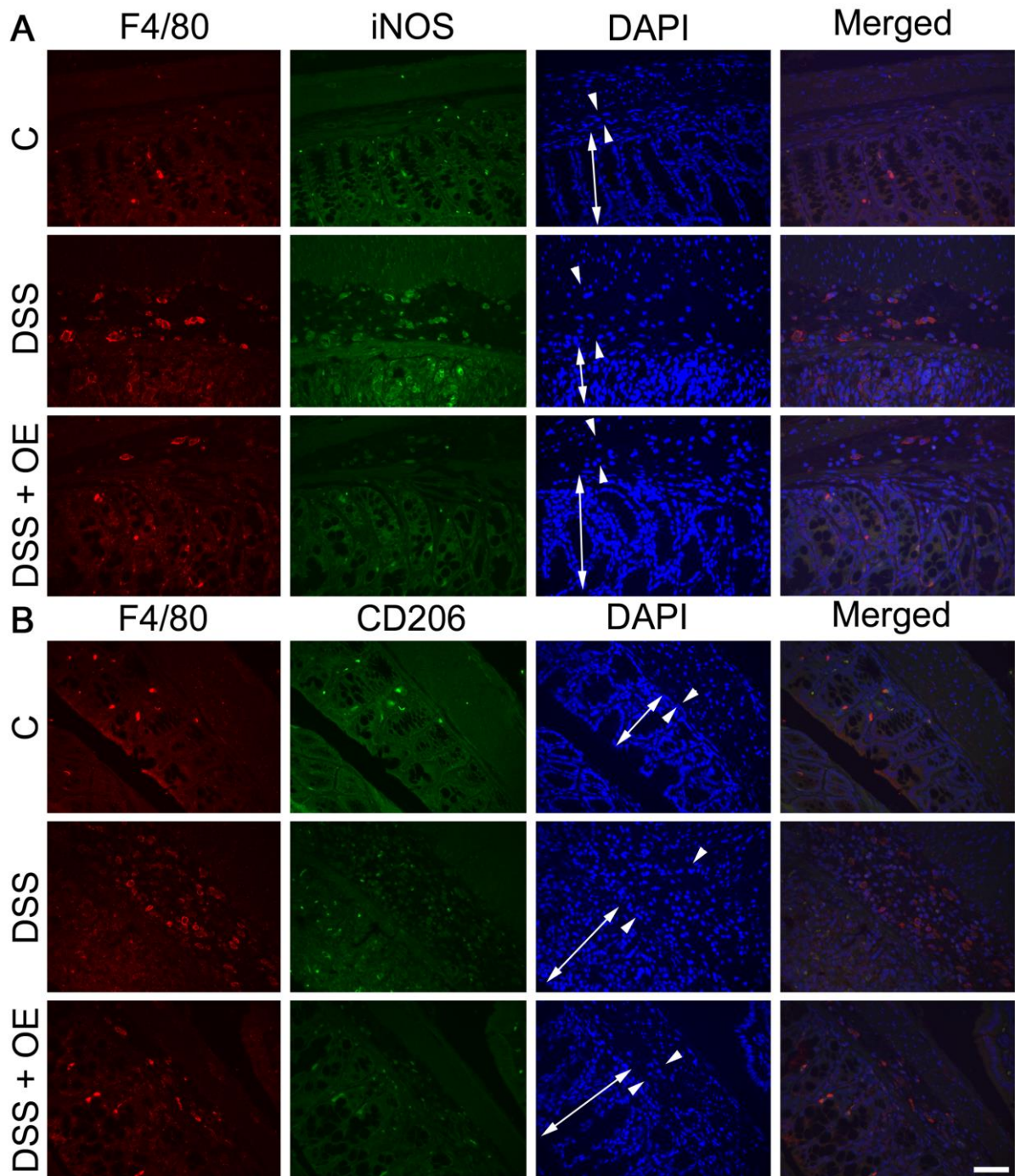


Figure 3.8 Effect of enoxaparin on macrophage phenotypes.

Co-immunostaining of macrophages and their phenotypes. Representative images of (A) M1 macrophages (F4/80⁺ and iNOS⁺) and (B) M2 macrophages (F4/80⁺ and CD206⁺) using colon tissue from n = 3 - 5 mice. F4/80 positive cells were visualized using Alexa Fluor® 594-conjugated goat anti-rat IgG (red) and iNOS or CD206 positive cells using Alexa Fluor® 488-conjugated goat anti-rabbit IgG (green). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Localization of mucosa (double-headed arrow) and submucosa (arrowheads) is indicated. Scale bar = 50 μ m for 400 \times magnification. Control, C; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE.

Quantitative analysis of these histological results showed that overall, a healthy colon contained twice as many M2 compared to M1 macrophages (open bars, Fig. 3.9). In contrast, the M1/M2 ratio increased markedly in DSS-induced colitis, with 92.0 ± 5.9 % of macrophages being M1 while only a minority were M2 cells (black bars, Fig. 3.9). On the other hand, oral enoxaparin treatment significantly increased the levels of M2 macrophages by 31.3 % ($p = 0.03$), whereas the M1 cells were reduced by 30.9% ($p = 0.0007$) compared to untreated colitis colons (grey bars, Fig. 3.9).

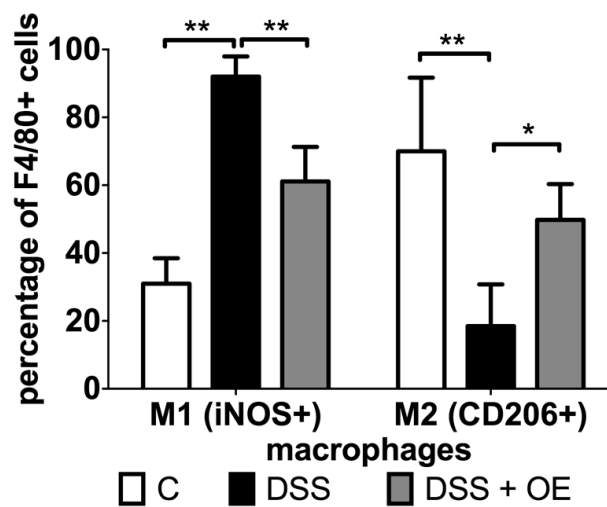


Figure 3.9 Effect of enoxaparin on macrophage phenotypes.

Quantification of macrophages expressing M1 and M2 phenotype markers in the colon. Results are expressed as percentage of double-positive macrophages from total macrophages \pm SD of five representative high-power fields per tissue section of $n = 3 - 5$ mice each. * $p < 0.05$ and ** $p < 0.01$.

IL-1 β acts as central mediator of pro-inflammatory immune responses. We therefore investigated a possible mechanistic link between reduced IL-1 β -levels and reduced M1 macrophage numbers in enoxaparin-treated mice (Fig. 3.10). Relative to healthy mice, IL-1 β immune-labelling increased under conditions of untreated colitis. At the same time, in the enoxaparin-treated colon, we observed a reduced expression of IL-1 β confirming our previous results (Fig. 3.5). However, using co-localization of IL-1 β staining with detection of a macrophage marker (F4/80), we were unable to demonstrate that IL-1 β was expressed by macrophages in this disease model (Fig. 3.10).

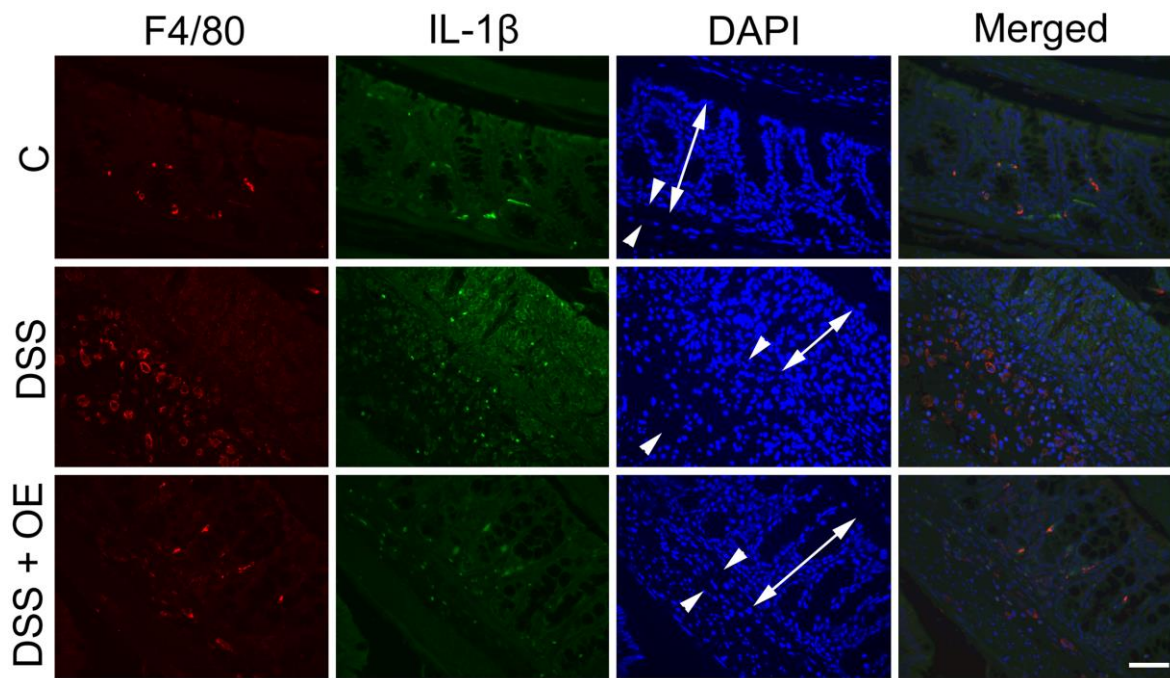


Figure 3.10 Effect of enoxaparin on IL-1 β expression on colon tissues.

Co-immunostaining of macrophages and IL-1 β . Representative images of IL-1 β and F4/80 staining of colons from $n = 3 - 5$ mice. F4/80 positive cells were visualized using Alexa Fluor® 594-conjugated goat anti-rat IgG (red) and IL-1 β positive cells using Alexa Fluor® 488-conjugated goat anti-rabbit IgG (green). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Localization of mucosa (double-headed arrow) and submucosa (arrowheads) is indicated. Scale bar = 50 μ m for 400 \times magnification. Control, C; untreated colitis, DSS; colitis with oral enoxaparin, DSS+OE.

3.3.7 Orally administered enoxaparin retains occludin and claudin-4 by attenuating crypt loss

The intact intestinal epithelium serves as a protective layer, which is maintained by the intercellular interactions between tight junction proteins such as occludin and claudins [13]. Immunohistochemistry was performed to determine the localization of tight junction proteins, occludin and claudin-4. As shown in Figure 3.11, occludin and claudin-4 proteins were homogenous as well as continuous on colonic epithelial cell membrane in healthy control. In contrast, in sections with untreated colitis, massive mucosal erosion and crypt loss prevented the reliable detection of tight junction proteins (Fig. 3.11). However, in mice

receiving oral enoxaparin, colonic occludin and claudin-4 staining indicated a greater proportion of intact colonic crypts (Fig. 3.11). On the other hand, intraperitoneal enoxaparin resulted in a tight junction protein staining comparable to untreated colitis mice, while enoxaparin treatment in healthy mice via the diet or by intraperitoneal injection did not alter the distribution or quantity of tight junction proteins in the colon.

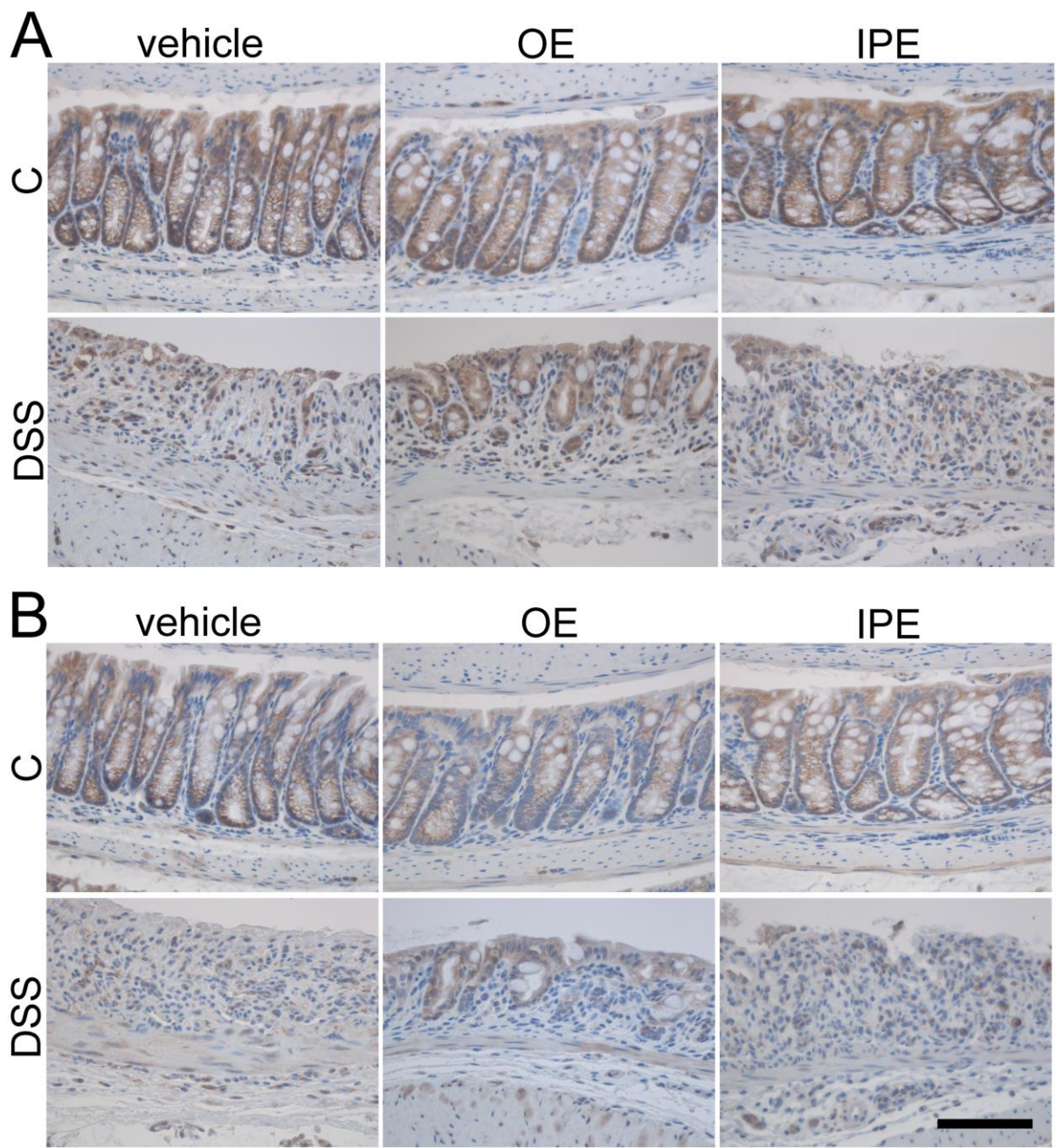


Figure 3.11 Effect of enoxaparin on epithelial tight junctions of colon.

Immunostaining of (A) occludin and (B) claudin-4 in the distal colon of healthy and colitis mice treated with and without enoxaparin. Scale bar = 100 μ m for 400 \times magnification. Control, C; untreated colitis, DSS; oral enoxaparin, OE; intraperitoneal injection of enoxaparin, IPE.

3.4 Discussion

In this study, we have described the therapeutic potential of the LMWH, enoxaparin, for the amelioration of acute colitis. Oral enoxaparin reduced the severity of clinical activity, histological damage and the immunological response associated with colitis. To our knowledge, this is the first report to demonstrate that enoxaparin given through the diet is highly effective in ameliorating experimental colitis, while inducing no mortality and adverse effects in healthy mice. Consistent with other study [65], our results showed that enoxaparin given as intraperitoneal injection did not reduce the severity of colitis. To date, animal studies investigating the efficacy of different doses of subcutaneously administered enoxaparin have reported inconsistent results. For example, subcutaneous enoxaparin at 80 μ g/kg/day over 7 days effectively ameliorated experimental colitis, while this effect was not observed at lower (40 μ g/kg/day) or higher (200 μ g/kg/day) doses in the same study [73]. Likewise, positive effects for subcutaneous enoxaparin at doses of 250-280 μ g/kg/day have been reported [66,97], while enoxaparin at 3 mg/kg/day was shown to be less efficacious [95]. Overall, our results are comparable to previous studies showing that enoxaparin is more effective when administered orally and not parenterally. Oral enoxaparin was previously reported to reduce the severity of colitis [94,95], while on the other hand intraperitoneal enoxaparin at a dose of ten times higher (5 mg/kg/day) [65] than our study (0.5 mg/kg/day) also did not relieve colitis-associated symptoms in mice, in agreement with our results. One limitation of prior studies is the uncertainty around the local availability of drug in the intestine when delivered subcutaneously or intraperitoneally, which is further complicated by observations that subcutaneous administration of enoxaparin produced inconsistent

results at different doses as described above. At present it is unclear if the reported discrepancies are a result of different dosing regimens or if they are related to the use of different pre-clinical colitis models. Overall, our results, together with the reports described above, strongly suggest that after parenteral administration, LMWHs cannot reliably reach areas of inflammation at the intestinal mucosa or submucosa. In contrast, current evidence supports the idea that oral administration is effective to deliver enoxaparin locally. From a patient's perspective, this is not only the preferred option, but this route of administration is likely to restrict the systemic availability of LMWHs, which therefore reduces the risk of bleeding complications. We did not test the stability of enoxaparin in the food mixture after storage in freezer (a freezing/thawing cycle). However, Patel *et. al* (2009) had demonstrated that slow freezing to - 196 °C with rapid thawing, or rapid freezing with slow thawing, resulted in only negligible loss of anti-factor Xa activity of enoxaparin [144].

Previously, the disease activity index was commonly used to summarise three clinical scores of colitis [95,97]. In this study however, we evaluated individual clinical parameters on a daily basis to identify differences of colitis severity and to better understand treatment-specific responses. Using this approach, oral enoxaparin showed a significant protection against weight loss, diarrhoea and intestinal bleeding as well as attenuation of histological features of colitis.

A multitude of cytokines is implicated in the pathogenesis of UC [15]. These mediators form a complex network that regulates mucosal inflammation and affect the integrity of epithelium [15]. Since pro-inflammatory cytokines from patient samples correlate with disease activity [240,244], treatments that modulate these mediators are likely to be of therapeutic use. In our study, we quantified a larger number of cytokines compared to previous studies to get a broader understanding of the immunological cytokine response

during intestinal inflammation and especially in response to enoxaparin treatment. The presence of elevated pro-inflammatory cytokines during intestinal inflammation was in general agreement with previous studies [243,245,246]. Although these studies reported on a significantly lower number of cytokines, overall they mirrored our results of a broad pro-inflammatory environment [245,246]. Also, in our study, oral enoxaparin reduced the levels of a number of mucosal cytokines including IL-1 α , IL-1 β , IL-10, MIP-1 α , MIP-1 β , G-CSF, GM-CSF during colitis. The importance of individual cytokine levels is reflected by their good correlations with the colitis-induced body weight loss of each animal.

Macrophages found in the colon of active UC patients are key mediators of human UC [247] and induce tissue damage by secreting inflammatory cytokines in the colonic mucosa during acute colitis [243]. Discrete macrophage subsets with divergent effects are often grouped functionally as M1 (monocyte-derived or classically activated or pro-inflammatory) and M2 (tissue-resident or alternatively activated or anti-inflammatory) macrophages [247-249]. During intestinal inflammation, monocytes are recruited and differentiated into pro-inflammatory macrophages within the lamina propria under the influence of GM-CSF [247]. This growth factor is necessary for the development, differentiation and proliferation of monocyte-derived inflammatory macrophages and is known to contribute to M1 polarization [248]. As expected by the reduced levels of GM-CSF in response to oral enoxaparin treatment, reduced numbers of M1 macrophages and elevated numbers of M2 macrophages were detected. These results are in agreement with other reports where M1 macrophages contributed critically to DSS-induced colitis, while M2 macrophages were protective [250,251]. Although our data suggest that that oral enoxaparin effectively reduced GM-CSF level, which could in part be responsible for suppressed M1/M2 ratio, this observation has to be confirmed in detailed future studies. Since macrophages are a major source of other pro-inflammatory cytokines, this connection

could also explain the reduction of multiple other cytokines such as IL-1 α , IL-1 β , IL-10, MIP-1 α and MIP-1 β that originated from inflamed colon tissues. However, the precise source of cytokines in this model of colitis is not completely known at present and these mediators could in principle be secreted by a variety of cells during inflammation. In human IBD, it is thought that IL-1 β is expressed by macrophages in the inflamed colon [252]. In contrast, in our mouse model, we were unable to demonstrate IL-1 β expression in intestinal macrophages. Together with the strong reduction of IL-1 β in colon tissue in response to enoxaparin, this suggests that enoxaparin either does not only target macrophages or that the observed changes to IL-1 β level are a secondary effect. In contrast to previous observations [73,94], the expression of TNF- α was not significantly affected by enoxaparin treatment. TNF- α is thought to play a significant role in inflammatory cellular signalling which is reflected by the successful clinical use of TNF- α inhibitors in UC patients [229]. However, it is likely that a number of factors including disease location, type of inflammation, pathogenic mechanisms and levels of multiple cytokines in combination affect the response to colitis treatment [15]. Therefore, agents that target a single pro-inflammatory cytokine are likely limited in their ability to offer an effective maintenance therapy over extended periods of time, while simultaneously modulating multiple pro-inflammatory mediators to reduce maturation and infiltration of immune cells could provide a more sustainable strategy against inflammation.

Epithelial barrier integrity is essential to a healthy gut function [8] and our understanding of how epithelial homeostasis is altered in response to intestinal inflammation is indispensable to develop therapeutic interventions that facilitate mucosal healing and normalize epithelial functions. In UC, the reduction of tight junctions, associated with increased intestinal permeability and impaired epithelial function [253-255] is at least partially caused by chronic inflammation [8]. In line with our results, previous studies

reported a disrupted and irregular expression pattern of tight junction proteins including occludin and claudin-4 in the colonic mucosa in mice with DSS-induced colitis [256,257]. However, in this study, DSS-treatment led to massive epithelial damage with loss of epithelial cells that prevented us to reliably quantify tight junction proteins. Nevertheless, both tight junction proteins could act as surrogate markers to indicate the effect of oral treatment as it correlates with the retention of crypt architecture. Since it is likely that oral enoxaparin mainly acts locally to relieve the severity of colitis [95], it is conceivable that its interaction with epithelial cells could directly protect them against DSS-induced damage. Protecting epithelial integrity would retain the epithelial barrier function and protect against infiltration of microbial antigens into lamina propria. The retention of crypt architecture could be the main reason for the localized and regular staining of tight junction proteins in colitis mice treated with oral enoxaparin, which is similar to the staining observed in healthy mice.

Although heparins are well-known for their non-anticoagulant effects [46], their exact mode of action in UC remain unclear. Different mechanisms including a reduced infiltration of leukocytes as well as pro-inflammatory mediators have been postulated [73,90,98]. Heparins are structurally similar to HS. Loss of cell surface HS proteoglycans is reported in patients with UC, resulting in decreased intestinal mucosal healing [57]. It has been also postulated that heparins can increase mucosal healing in UC or during colitis by substituting the loss of cell surface HS proteoglycans. Like other LMWHs and UFH, enoxaparin is also composed of a complex mixture of structurally unknown oligosaccharides. Further work to identify the specific components of enoxaparin that are responsible for the observed effects will be a pre-requisite to identify their mode(s) of action as well as to progress the most promising molecules towards clinical trials.

3.5 Conclusion

Overall, enoxaparin given at an early stage of colitis significantly prevents the development of colitis and reduces the pathology associated with acute colitis induced by DSS. This study extends our current understanding of oral administration of enoxaparin during acute colitis, which is a crucial step towards the use of enoxaparin for the treatment of colitis. The ability of enoxaparin to reduce inflammation and retain epithelial integrity along with the possibility of oral delivery to provide a better safety profile for clinical use, serves as a rationale to develop enoxaparin components as a therapeutic for patients with UC. Similar investigational approach using other heparin-like molecules such as fucoidans would be interesting to see if these molecules also display similar *in vivo* activity when used in animal model of colitis.

Chapter 4

Chapter 4 Fucoidan Extracts Ameliorate Acute Colitis

4 Abstract

Inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, are an important cause of morbidity and impact significantly on quality of life. Overall, current treatments do not sustain a long-term clinical remission and are associated with adverse effects, which highlight the need for new treatment options. Fucoidans are complex sulphated, fucose-rich polysaccharides found in edible brown algae and are described as having multiple bioactivities including potent anti-inflammatory effects. Therefore, the therapeutic potential of two different fucoidan preparations, fucoidan-polyphenol complex (Maritech *Synergy*) and depyrogenated fucoidan (DPF) was evaluated in the dextran sulphate sodium mouse model of acute colitis. Mice were treated once daily over 7 days with fucoidans via oral (*Synergy* or DPF) or intraperitoneal administration (DPF). Signs and severity of colitis were monitored daily before colons and spleens were collected for macroscopic evaluation, cytokine measurements and histology. Orally administered *Synergy* and DPF but not intraperitoneal DPF treatment, significantly ameliorated symptoms of colitis based on retention of body weight, as well as reduced diarrhoea and faecal blood loss, compared to the untreated colitis group. Colon and spleen weight in mice treated with oral fucoidan was also significantly lower, indicating reduced inflammation and oedema. Histological examination of untreated colitis mice confirmed a massive loss of crypt architecture and goblet cells, infiltration of immune cells and oedema, while all aspects of this pathology were alleviated by oral fucoidan. Importantly, in this model, the macroscopic changes induced by oral fucoidan correlated significantly with substantially decreased production of 15 pro-inflammatory cytokines by the colon tissue. Overall, oral fucoidan

preparations significantly reduce the inflammatory pathology associated with DSS-induced colitis and could therefore represent a novel nutraceutical option for the management of IBD.

4.1 Introduction

Inflammatory bowel disease (IBD) is a known medical burden in most developed countries and a significant cause of morbidity [228]. Due to the nature of chronic inflammation involving the gut mucosa, patients present with symptoms such as abdominal pain, diarrhoea, bloody stool and weight loss, which significantly decreases their quality of life [2,3]. Currently, pharmacological and surgical interventions are the two main management approaches for IBD. Currently available drugs, which aim to decrease inflammation, show limited effectiveness for long term remission and are associated with side effects [36]. Monoclonal antibodies that inhibit TNF- α such as infliximab have shown clinical usefulness but at a relatively high cost [258,259]. Furthermore, about 20 - 40 % of patients do not respond to TNF- α inhibitors, while in up to 50 % of patients, the therapeutic response is lost after 1 - 2 years [39,260,261]. Surgery is reserved for about 20 - 30 % of patients who are unresponsive to medication and develop life-threatening complications such as perforation, refractory rectal bleeding and toxic megacolon [2,3]. Even after surgery, patients are predisposed to the risk of postoperative complications such as bowel obstruction, anastomotic strictures, pouchitis, sexual dysfunction and pouch failure [2,3]. Therefore, there is an urgent need for new treatment options that are safe, able to sustain clinical remission and improve mucosal gut healing.

Animal models have become a useful tool to study the pathophysiology of IBD and to test the *in vivo* efficacy of potential therapeutic agents [262]. A number of chemically-induced colitis models have been described that use different colitogenic substances, such as dextran sulphate sodium (DSS), di- or tri-nitrobenzene sulfonic acid, oxazolone or acetic acid [235]. DSS-induced colitis in mice is the most commonly used model; it is associated with severe epithelial damage and a robust inflammatory response in the colon [263,264].

DSS-treated mice show signs of acute colitis including rectal bleeding, body weight loss, passage of fresh blood through the anus, usually in or with stools and diarrhoea [263,265]. Macroscopic and histological examination reveals shortening of the intestine, submucosal ulceration and loss of goblet cells and crypt structure, as well as infiltration of large numbers of inflammatory cells into the colonic mucosa [239,263,265]. These macroscopic and histopathological changes are associated with the excessive production of pro-inflammatory cytokines [266,267]. Although this animal model has limitation regarding the accurate representation of the clinical course of IBD patients, it nevertheless recapitulates the common clinical features of inflammation and histopathology seen in human disease [263]. Importantly, despite its shortcomings, this model has been validated through the use of several clinically used therapeutic agents against IBD [267].

Given the need for new treatment options for IBD, we have tested two fucoidan extracts in the DSS-induced colitis model. Fucoidans are a class of sulphated, fucose-rich polymers found in edible brown macroalgae and echinoderms, which are commercially available as dietary supplements. Fucoidans exhibit different bioactivities, which have been reported in many pre-clinical *in vitro* and *in vivo* models [156]. However, the efficacy and characteristics of these bioactivities can vary significantly and depend on the source, species, MW, composition and structure of the molecules, as well as on the route of administration. The reported bioactivities of fucoidans are diverse and include anticoagulation, blocking of lymphocyte adhesion and invasion, inhibition of multiple enzymes, induction of apoptosis, antiviral activity and, most importantly for this study, a substantial anti-inflammatory activity [156,166,172]. Specifically, current evidence that fucoidans can be beneficial for regulating gut health is derived from a variety of pre-clinical models and studies. In a mouse model of chronic colitis orally delivered fucoidan (from *Cladosiphon*) was reported to downregulate the levels of the pro-inflammatory cytokine IL-6, which was also confirmed

through *in vitro* studies [167]. Similarly, in a rat model of acute colitis, an orally delivered polysaccharide food supplement containing fucoidan reduced monocyte numbers and improved clinical markers of colitis [268]. Fucoidan is also known to protect against aspirin-induced gastric ulcers in a rat model [269] and is known from pre-clinical and clinical investigations to inhibit gastric pathogens [270-272].

Fucoidan extracts are commercially available as dietary supplements and for this study, two preparations of fucoidan from *Fucus vesiculosus* were compared. One is a highly purified preparation of fucoidan (depyrogenated fucoidan, DPF) with a relatively low MW, whereas the other (Maritech *Synergy*) is a fucoidan that is naturally complexed with polyphenols. We demonstrate here for the first time that orally delivered fucoidan or a fucoidan polyphenol complex both significantly suppress the inflammatory response in the acute DSS-induced model of colitis. These findings could open the way towards new treatment options for patients with IBD.

4.2 Materials and methods

4.2.1 Animal colitis model

All animal experiments were approved by the Committee of Animal Ethics of the University of Tasmania (A13576) and were conducted in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (8th Edition 2013). Male C57BL/6 mice (aged 8 - 10 weeks; 21 - 27 g, average weight \approx 25 g) were obtained from the University of Tasmania animal breeding facility and housed in a temperature-controlled, non-sterile environment under a 12-hour day/ night light cycle. Mice derived from different litters and at different time points over a period of three weeks were randomised into the different treatment arms based on body weight and age to ascertain comparable group compositions. Body weights of mice were assessed daily over an initial acclimation period

of one week. All mice were non-fasting and had access to food and drinking water (autoclaved tap water) *ad libitum*. Colitis was induced by supplementing 3 % w/v of dextran sulphate sodium (DSS, MW = 40,000-50,000, USB, Affymetrix Inc, Ohio, USA) in the drinking water of mice from day 1 to day 8 (day of termination). Control mice received drinking water without DSS.

4.2.2 Formulation of fucoidan extracts

Depyrogenated fucoidan (DPF) and fucoidan-polyphenol complex (Maritech *Synergy*), extracts (Table 4.1) from marine brown seaweed *Fucus vesiculosus* (bladder wrack) were provided by Marinova Pty Ltd, Tasmania, Australia.

Table 4.1 Fucoidan extract composition*

Extract	Fucoidan					
	Neutral Carbohydrates (%)	Sulphates (%)	Counter-ions (%)	Poly-phenols (%)	Uronic acids (%)	Peak MW (kDa)
Fucus-polyphenol (Maritech <i>Synergy</i>)	40.2	21.8	6.3	26.2	3.6	203.1
High-purity fucoidan (DPF)	59.5	26.6	11.9	< 0.5	1.4	61.7

*Data provided by Marinova Pty Ltd

Both extracts are polydisperse, containing fractions from 5 kDa to above 1000 kDa. Fucus-polyphenol is the more polydisperse and features a greater proportion of high molecular weight molecules. The major component of the neutral carbohydrate content of both compounds is fucose. The fucus-polyphenol extract (Maritech *Synergy*) contains 25.8 % fucose overall, whilst the high-purity fucoidan (DPF) contains 53.3 % fucose.

DPF injection solution (5 mg/ml) was prepared by dissolving DPF in water for injection and filtered using a 0.45 µm filter. Fucoidan-containing food mash was prepared as described previously [273]. Briefly, 0.75 g of fucoidan extract (DPF or *Synergy*) and 9 g of sucrose (4 % w/w of food mash) were separately well-suspended/ dissolved in autoclaved

water before they were homogenously mixed into a solution with a final weight of 105 g. Food powder (120 g) was then dispersed slowly into the mixture with stirring to make a food paste. The food mash was aliquoted as 3 g/dish and stored at -20 °C.

4.2.3 Treatment with fucoidan extracts

Healthy control (HC) mice received standard chow while another group which received DSS in their drinking water and only vehicle through the food represented the disease control (DSS). Three treatment groups (n = 10 mice/ group), where all mice received DSS in their drinking water, were randomised for treatment with fucoidan extracts for 7 days (day 1 - day 7). The first treatment group was injected intraperitoneally with DPF injection solution (IPDPF, 0.25 mg/ mouse, 10 mg/kg/day). The second and third treatment groups were given oral DPF and Maritech *Synergy* respectively (ODPF and OS; 10 mg/ mouse, 400 mg/kg/day). For the oral treatment groups, the mice were single-caged throughout the experiment to ascertain the defined daily intake of fucoidan from prepared food mash. In addition, all mice had access to normal food pellets *ad libitum*.

4.2.4 Evaluation of intestinal inflammation

Mice were weighed and observed daily for stool consistency and the presence of blood in the stool and sign of gross bleeding on the anus site [235]. Stool samples were obtained from individual mice and were tested by using Hemocult II slides (Beckman Coulter Inc., California, USA).

4.2.5 Termination of experiment and tissue sampling

Mice were sacrificed on day 8 by carbon dioxide inhalation followed by cervical dislocation. Upon dissection, the spleen was removed and weighed. The full colon was then carefully

removed. Colon length was measured between the ceco-colic junction and the proximal rectum. Subsequently, the entire colon was opened longitudinally to remove the faecal content before the wet weight of each colon was measured. The colon was then divided longitudinally to be used for either fixation in 10 % v/v buffered formalin and histological analysis or for tissue explant culture.

4.2.6 Histologic evaluation of colitis

Fixed colons were processed into paraffin blocks and were cut into 4 μ m sections, which were stained with hematoxylin and eosin. Subsequently, all sections were graded in an investigator-blinded manner regarding the severity of the tissue damage using light microscopy (Leica DM2500, Leica Microsystems Pty Ltd, NSW, Australia and Leica application suite version 3 software) at 100 \times / 400 \times magnification. The severity of colitis was assessed according to the criteria described previously [236-238].

4.2.7 Tissue explant culture and measurement of cytokine levels

Tissue from the distal colon was cut and washed with cold PBS before each sample was transferred into a 12 well-plate containing 1 ml/ well of RPMI 1640 culture medium (In Vitro Technologies Pty Ltd, Victoria, Australia) supplemented with 10 % v/v foetal calf serum (FCS, Gibco, Life Technologies, Victoria, Australia), penicillin (100 mU/L) and streptomycin (100 mg/L) (Sigma-Aldrich Pty Ltd, NSW, Australia). After 24 hours of incubation, supernatants were collected and stored at -80 °C until further analysis. A Bio-Plex Pro Mouse cytokine 23-plex kit (Bio-Rad Laboratories, Inc., California, USA) was used to determine the cytokine levels in the tissue culture supernatants. Following the manufacturer instructions, cytokine standards and undiluted culture supernatants were tested in duplicate on a Bio-Plex 200 instrument equipped with Bioplex Manager software, version

6 (Bio-Rad Laboratories, Inc., California, USA). The cytokine levels were normalized by dividing the cytokine results (pg/ml) by the measured tissue weight (mg) and expressed as pg/ml cytokine/ 10 mg of tissue.

4.2.8 Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 6, GraphPad Software Inc, CA, USA). Statistical significance was evaluated using one or two way analysis of variance (ANOVA), followed by a multiple comparison test: Dunnett's test or Tukey's test. Pearson's correlation coefficient (r^2) was determined for the relationship between two variables when necessary. A p value of < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Oral fucoidan extracts ameliorate colitis-induced weight loss

Mice receiving DSS in the drinking water developed acute colitis, evidenced by a significant reduction of body weight, on day 8 (-12.3 ± 4.3 %; $p < 0.0001$) (Fig. 4.1A). Oral administration of both fucoidan extracts significantly reduced body weight loss compared to untreated colitis (Fig. 4.1A). Statistically significant differences were obtained on day 7 ($p = 0.05$) and day 8 ($p < 0.0001$), with a maximum weight loss of only 5.9 ± 4.4 % for mice treated with oral DPF (ODPF). Likewise, mice treated with oral *Synergy* (OS) only showed a weight loss of 7.8 ± 3.1 % on day 8 which was significantly different to untreated mice ($p = 0.0005$). There was no significant difference between the effectiveness of the oral fucoidan extracts. In contrast, intraperitoneal (i.p) DPF (IPDPF) showed no protection but resulted in significantly increased weight loss on day 8 (-17.5 ± 2.1 %, $p = 0.001$) compared to the untreated mice.

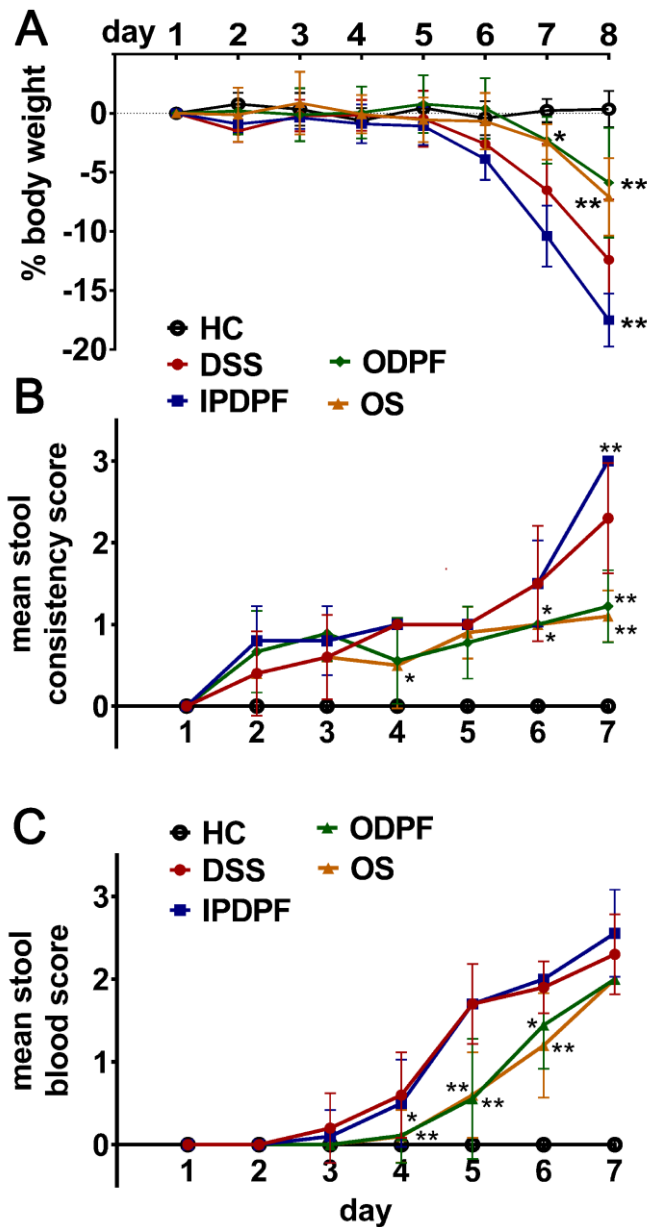


Figure 4.1 Effect of fucoidan extracts during acute colitis.

(A) Daily changes of body weight during colitis induction in C57BL/6 mice with and without fucoidan extracts treatment versus healthy control. Body weight changes, expressed as percentage, were calculated by dividing body weight on each day with the initial body weight before the start of DSS treatment. Stool samples were scored for (B) consistency and (C) occult blood on a daily basis during experiment. Data represent percentage or mean \pm SD of $n = 6 - 10$ animals. Significance is indicated by * $p < 0.05$ and ** $p < 0.01$ using two-way ANOVA followed by Tukey's post-test. Healthy control, HC; untreated colitis, DSS; intraperitoneal injection of depyrogenated fucoidans, IPDPF; oral treatment of depyrogenated fucoidans, ODPF; oral treatment of Maritech *Synergy*, OS.

4.3.2 Oral fucoidan extracts delay the development of diarrhoea and the appearance of blood in stool

After exposure to DSS, untreated mice showed clear changes in stool consistency (Fig. 4.1B) and the presence of blood in stool (Fig. 4.1C) compared to mice without exposure to DSS. As early as day 2, mice receiving DSS developed soft stool that still contained formed pellets. On day 3, stool of untreated mice with DSS tested positive for occult blood. The severity of symptoms progressively increased towards the end of the experiment, when mice had bloody soft stool or diarrhoea. Consistent with retention of body weight, mice which received oral DPF (ODPF) or *Synergy* (OS) showed significant protection against diarrhoea over the entire observation period (Fig. 4.1B). Oral fucoidan extracts also significantly protected against the presence of blood in stool from day 4, although this protection was lost at day 7 (Fig. 4.1C). Intraperitoneal DPF (IPDPF) was not effective in alleviating DSS-induced blood in stool (Fig. 4.1C) or worsening of stool consistency (Fig. 4.1B).

4.3.3 Oral fucoidan extracts protect against changes to colon and spleen

After the observation period, DSS-induced changes to colon and spleen were analysed. DSS-treated mice showed significantly shorter (6.4 ± 0.5 cm; about 77 %) compared to healthy control mice (8.3 ± 0.7 cm) (Fig. 4.2A). Although protection by oral fucoidan did not reach significance compared to untreated mice, there was a trend towards reduced colon shortening for ODPF and OS (6.9 ± 0.6 cm and 7.0 ± 0.5 cm; respectively; about 84 % of healthy controls). Wet colon weight, an indicator of intestinal oedema and inflammation, was presented as the ratio of colon weight over body weight (mg/g). As expected, the untreated colitis group showed the highest relative weight (9.4 ± 1.2 mg/g) (Fig. 4.2B). The relative colon weight was significantly reduced by 19.1 % for ODPF (7.7 ± 0.9 mg/g; $p = 0.01$) and 16.5 % for OS (7.9 ± 1.0 mg/g; $p = 0.03$) compared to the untreated group (Fig. 4.2B).

Consistent with the previous results, IPDPF showed no effect with regards to colon length (6.5 ± 0.3 cm) (Fig. 4.2A) or relative colon weight (8.9 ± 1.5 mg/g) (Fig. 4.2B).

Intestinal inflammation is associated with spleen enlargement [264] and as expected, the relative spleen weight of the mice receiving DSS in our study was significantly increased (5.1 ± 2.0 mg/g; $p = 0.006$) compared to spleens from healthy control mice ($n = 6$) (Fig. 4.2C). Oral administration of either DPF or *Synergy* protected against the increased relative spleen weight (3.4 ± 0.5 mg/g and 3.4 ± 0.9 mg/g, respectively; $p = 0.009$) compared to untreated colitis mice, whereas IPDPF did not affect relative spleen weight (4.2 ± 0.5 mg/g, $p = 0.28$) (Fig. 4.2C).

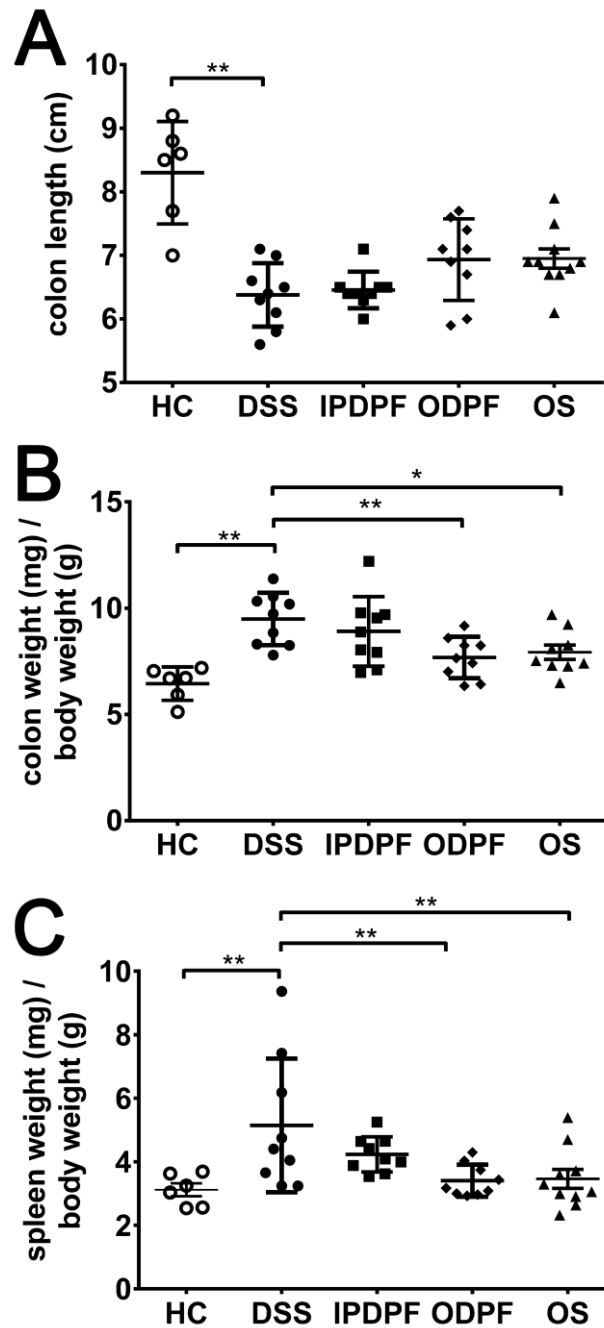


Figure 4.2 Effect of fucoidan extracts on colon and spleen.

Colons were measured for their length (A) and weight. Spleens were also removed and weighed. The organ weights were then presented as a ratio of colon weight (B) or spleen weight (C) over body weight respectively. Data represent the mean \pm SD of $n = 6 - 10$ animals. Significance is indicated by * $p < 0.05$ and ** $p < 0.01$ using one-way ANOVA followed by Dunnett's post-test. Healthy control, HC; untreated colitis, DSS; intraperitoneal injection of depyrogenated fucoidans, IPDPF; oral treatment of depyrogenated fucoidans, ODPF; oral treatment of Maritech *Synergy*, OS.

4.3.4 Oral fucoidan extracts decrease colon damage and infiltration of inflammatory cells

Histological examination of colon tissue revealed that all DSS-treated mice displayed erosion or destruction of epithelium, crypt distortion, loss of goblet cells, submucosal oedema, increased colonic wall thickness and inflammatory cellular infiltration in the colon, mostly affecting the distal colon (Fig. 4.3A). While healthy mice showed no signs of histological colon damage (score 0), DSS resulted in cumulative damage scores of 8.1 ± 4.4 for the proximal colon (Fig. 4.3B) and 22.6 ± 4.3 for distal colon (Fig. 4.3C). Co-treatment with oral fucoidan extracts induced protection as evidenced by retention of colonic structure, reduced infiltration of inflammatory cells and reduced submucosa oedema (Fig. 4.3A), which overall resulted in a significant overall reduction of cumulative histological disease scores for the distal colon (14.4 ± 6.6 ; 36.3 %, $p = 0.005$ and 15.0 ± 5.6 ; 33.6 %, $p = 0.007$ for ODPF and OS respectively) (Fig. 4.3B and 4.3C). In contrast histology scores for the proximal colon demonstrated no significant protection by oral fucoidan extracts. IPDPF also showed no marked protection against distal or proximal colon damage, as indicated by high histological scores for the distal colon (22.7 ± 2.3) (Fig. 4.3C).

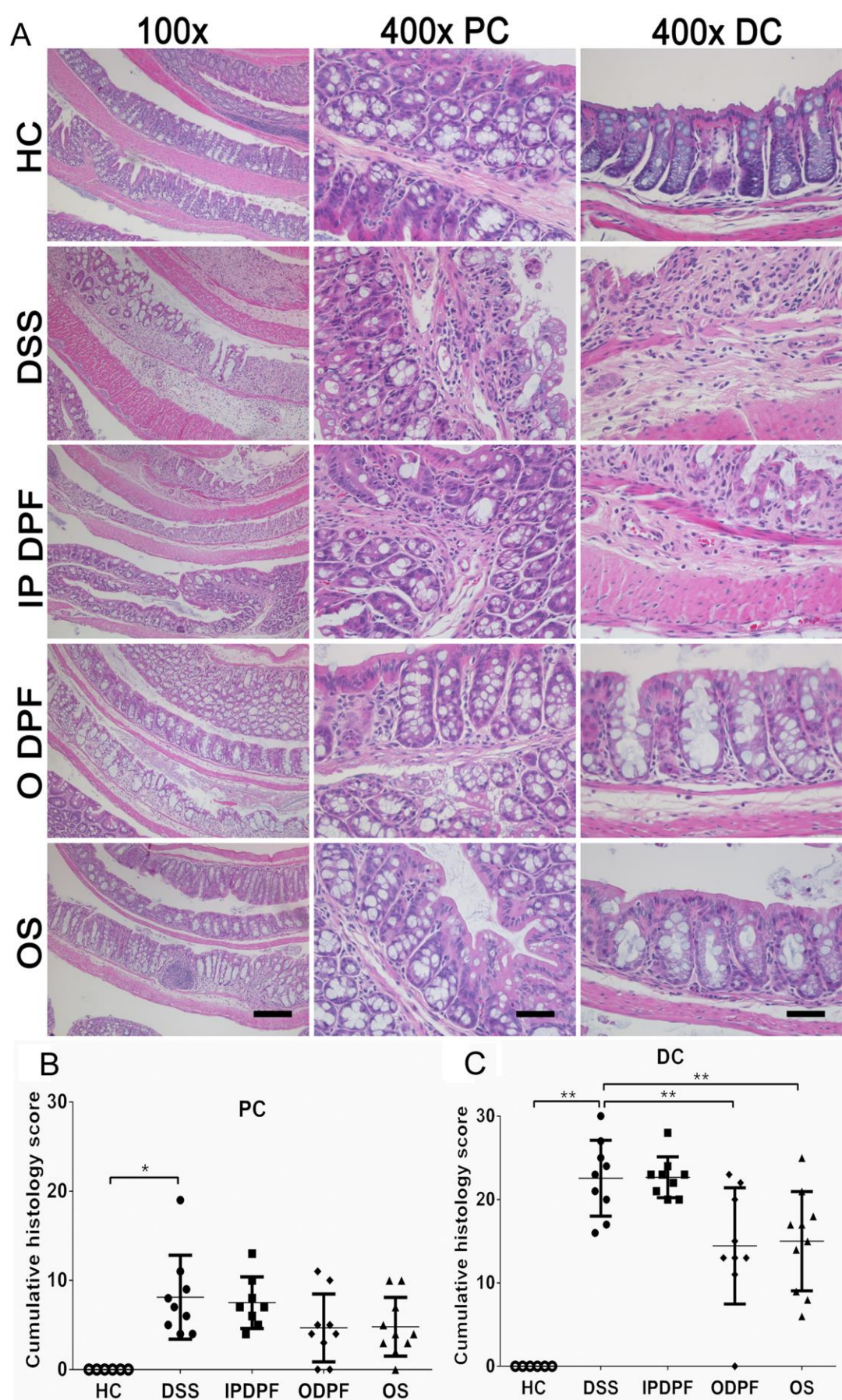


Figure 4.3 Effect of fucoidan extracts on colon histology.

(A) Representative hematoxylin and eosin stained colon sections of healthy controls, untreated mice with colitis and colitic mice that received fucoidan extracts. Cumulative histology damage scores for (B) Proximal colon (PC) and (C) Distal colon (DC). Data represent the mean \pm SD of $n = 6 - 10$ animals. Significance is indicated by * $p < 0.05$ and ** $p < 0.01$ using one-way ANOVA followed by Dunnett's post-test. Scale bars = 100 μm for 400 \times and 400 μm for 100 \times magnification. Healthy control, HC; untreated colitis, DSS; intraperitoneal injection of depyrogenated fucoidans, IPDPF; oral treatment of depyrogenated fucoidans, ODPF; oral treatment of Maritech *Synergy*, OS.

4.3.5 Oral fucoidan extracts reduce the levels of pro-inflammatory cytokines

Cytokines are important mediators of the mucosal immune response. We therefore quantified colon tissue-derived cytokine levels to characterize the extent of inflammation during the acute colitis in the DSS model and the effects of fucoidan extracts. DSS treatment induced a significant increase of a multitude of cytokines during the acute colitis period (Fig. 4.4). Compared to untreated colitis, both oral fucoidan extracts significantly lowered the levels of IL-1 α , IL-1 β , IL-10, MIP-1 α , MIP-1 β , G-CSF and GM-CSF (Fig. 4.4 and Table 4.2). In addition, oral *Synergy* also significantly reduced the levels of IL-3, IL-12 (p40), IL-12 (p70), IL-13, TNF- α and eotaxin (Fig. 4.4 and Table 4.2).

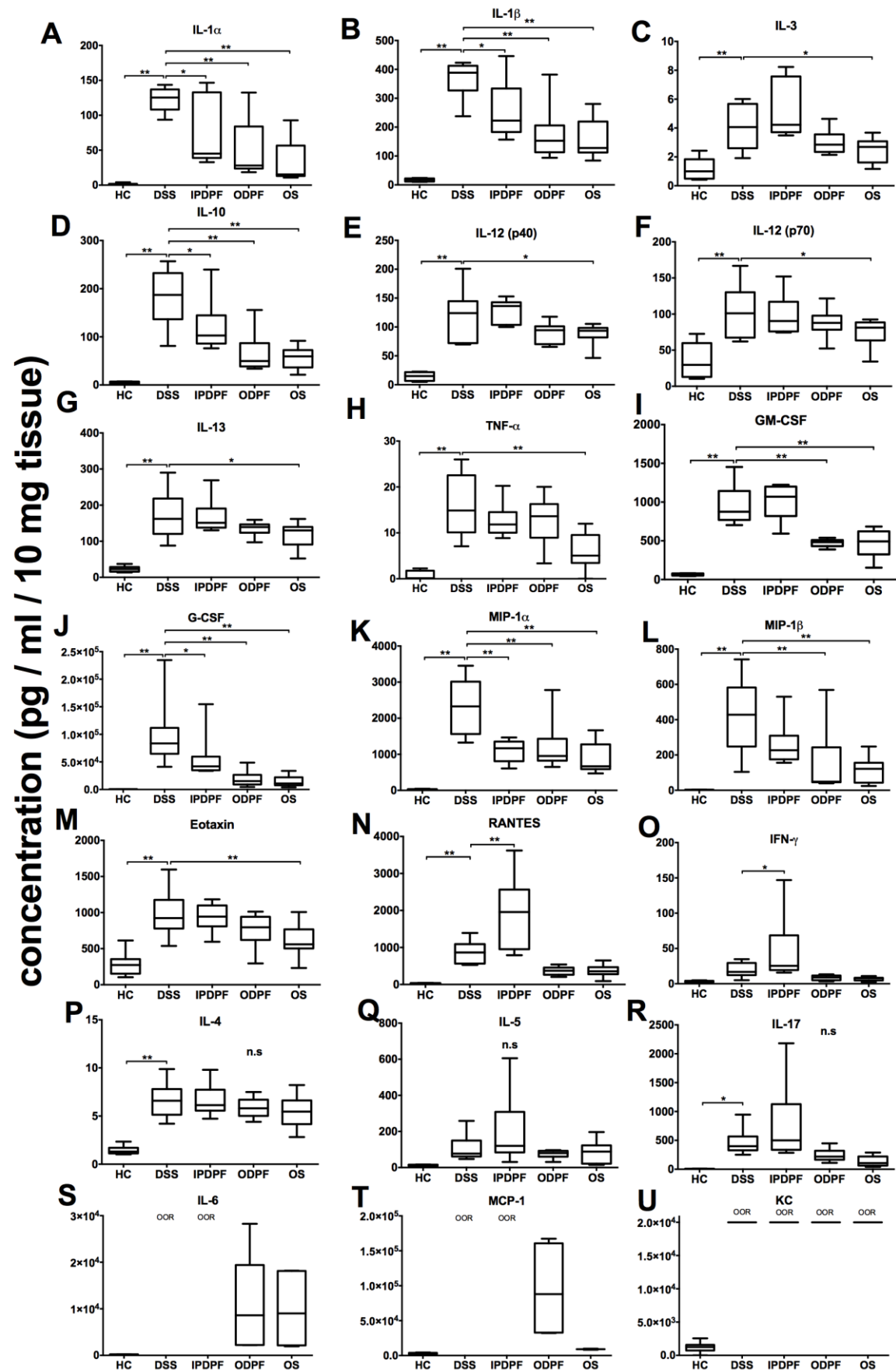


Figure 4.4 Effect of fucoidan extracts on colon-derived cytokine levels.

Distal colon tissue samples were cultured for 24 hours. The supernatants were assessed for cytokine levels using a Bio-Plex assay kit. Cytokine levels in the supernatant were normalized to tissue weight to obtain pg/ml of cytokines/ 10 mg of tissue. Data represent minimum, 25th percentile, median, mean, 75 percentile and maximum of cytokine levels of $n = 5$ animals. Significance is indicated by * $p < 0.05$ and ** $p < 0.01$ using one-way ANOVA followed by Dunnett's post-test. Concentrations fell out of range, OOR, exceeding upper detection limits; n.s, non-significant; interleukin, IL; monocyte chemotactic protein-1, MCP-1; keratinocyte-derived chemokine, KC; interleukin, IL; tumor necrosis factor- α , TNF- α ; granulocyte colony-stimulating factor, G-CSF; granulocyte-macrophage colony-stimulating factor, GM-CSF; macrophage inflammatory protein, MIP; regulated and normal T cells expressed and secreted, RANTES; interferon- γ , IFN- γ ; Healthy control, HC; untreated colitis, DSS; intraperitoneal injection of depyrogenated fucoidans, IPDPF; oral treatment of depyrogenated fucoidans, ODPF; oral treatment of Maritech Synergy, OS.

Table 4.2 Effect of fucoidan extracts on colon-derived cytokine levels.

Cytokine	Colon explant culture Treatment versus DSS					
	Intraperitoneal DPF		Oral DPF		Oral Synergy	
	% change	p value	% change	p value	% change	p value
IL-1α	-36.4	0.017	-55.9	0.0002	-71.3	< 0.0001
IL-1β	-28.6	0.016	-51.2	< 0.0001	-55.7	< 0.0001
IL-3	n.s		n.s		-50.7	0.0242
IL-10	-31.1	0.0308	-62.2	< 0.0001	-69.2	< 0.0001
IL-12 (p40)	n.s		n.s		-26.5	0.0386
IL-12 (p70)	n.s		n.s		-29.4	0.0398
IL-13	n.s		n.s		-30.8	0.0179
MIP-1α	-52.8	< 0.0001	-46.5	0.0004	-60.9	< 0.0001
MIP-1β	n.s		-60.0	0.0037	-72.7	0.0004
G-CSF	-43.7	0.0193	-80.6	< 0.0001	-85.2	< 0.0001
GM-CSF	n.s		-51.0	< 0.0001	-51.9	< 0.0001
Eotaxin	n.s		n.s		-36.6	0.0041
IFN-γ	85.6	0.0309	n.s		n.s	
TNF-α	n.s		n.s		-68.0	0.0003
RANTES	115.8	< 0.0001	n.s		n.s	

Supernatants from colon tissue explant culture were assessed for cytokine levels. The percent (%) change versus the DSS colitis group for each cytokine is shown. Not significant, n.s; interleukin, IL; macrophage inflammatory protein, MIP; granulocyte colony-stimulating factor, G-CSF;

granulocyte-macrophage colony-stimulating factor, GM-CSF; interferon- γ , IFN- γ ; tumor necrosis factor- α , TNF- α ; regulated and normal T cells expressed and secreted, RANTES.

Importantly, at the level of each individual mouse, the fucoidan-treatment-induced reduction of pro-inflammatory cytokine levels significantly correlated with the protection against body weight loss (Fig. 4.5). For two cytokines, IL-6 and MCP-1, DSS increased their levels beyond the upper detection limit of the assay, while co-treatment with both oral fucoidan extracts decreased their levels back into the measurable range (Fig. 4.4S and Fig. 4.4T). In contrast to the oral fucoidan formulations, the overall cytokine response to IPDPF was inconsistent. While IPDPF reduced the levels of IL-1 α , IL-1 β , IL-10, G-CSF and MIP-1 α , the levels of IFN- γ and RANTES were significantly increased by this treatment (Fig. 4.4 and Table 4.2).

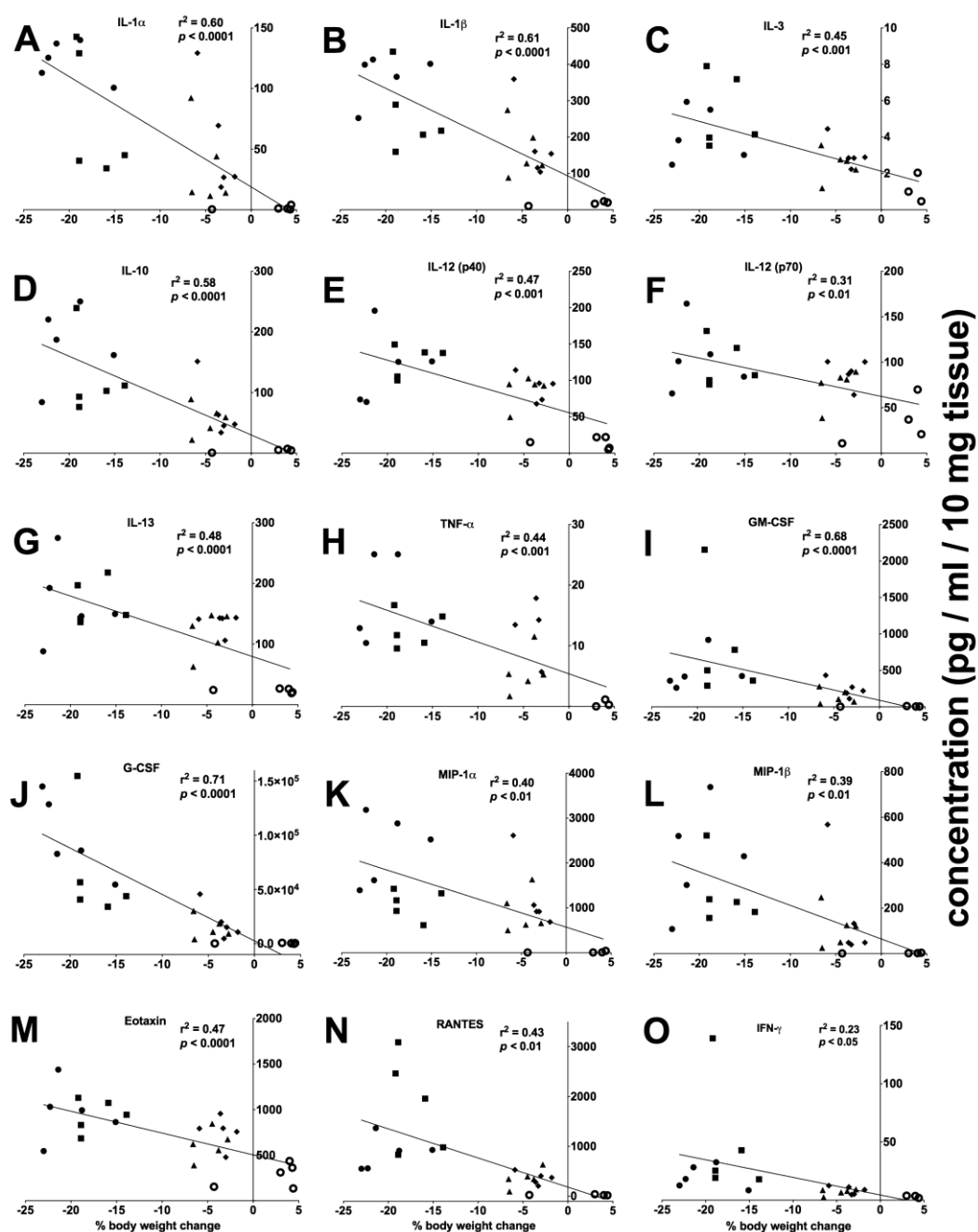


Figure 4.5 Correlation between colon-derived cytokine levels and treatment-induced changes to body weight.

Changes in cytokine levels by fucoidan extracts were correlated with the changes to body weight for individual mice. The value of Pearson correlation coefficient (r^2) is reported and significance is indicated by p value. Interleukin, IL; tumor necrosis factor- α , TNF- α ; granulocyte colony-stimulating factor, G-CSF; granulocyte-macrophage colony-stimulating factor, GM-CSF; macrophage inflammatory protein, MIP; regulated and normal T cells expressed and secreted, RANTES; interferon- γ , IFN- γ ; healthy control (open circle); untreated colitis (closed circle); intraperitoneal injection of depyrogenated fucoidan (closed square); oral treatment of depyrogenated fucoidan (closed diamond); oral treatment of Maritech Synergy (closed triangle).

4.4 Discussion

We have shown here that oral fucoidan extracts can effectively protect against DSS-induced acute colitis. Not only did the oral formulations alleviate the macroscopic pathologies such as body weight loss and stool consistency, they also significantly reduced the underlying intestinal inflammation. Although previous reports have demonstrated anti-inflammatory activities of fucoidan and fucoidan-containing extracts in different experimental models *in vitro* and *in vivo* [156,159,161,165,268,274-276], this is the first report to demonstrate that dietary fucoidan extracts from *Fucus vesiculosus* are highly effective in ameliorating experimental colitis through a consistent down-regulation of a significant number of pro-inflammatory cytokines.

Many different cytokines are involved in the pathogenesis IBD [277]. They form a complex network that regulates mucosal inflammation and affects the integrity of epithelium [15]. Since pro-inflammatory cytokines from patient samples correlate with disease activity [240,244], treatments that modulate these mediators are likely to be of therapeutic use. In our study, we quantified a larger number of cytokines compared to previous studies to get a broader understanding of the immunological cytokine response during intestinal inflammation and especially in response to fucoidan treatment.

Despite the numerous reports describing the different anti-inflammatory activities of fucoidans, there is no information available that would highlight a single mode of action that could be responsible for the effects observed in this study. It is now generally accepted that the inflammatory response in patients and animal models of IBD is predominantly macrophage driven [247,251,278]. During intestinal inflammation, monocytes are recruited and differentiated into macrophages within the lamina propria [247] and it is believed that the initial exposure of interstitial macrophages to bacterial antigens is responsible for the

activation of macrophages and that at least in the DSS model these activated macrophages subsequently stimulate the proliferation of T cells [279]. In particular the highest DSS-induced cytokines in our study, G-CSF and MIP-1 α , which were up-regulated by more than 200-fold and 100-fold respectively, support the strong involvement of macrophages in this model. On the other hand, fucoidans were reported to influence pro-inflammatory signalling molecules and pathways in macrophages, such as p38, Erk, JNK [165], HMGB1 and NF- κ B [163]. These signalling pathways are essential for macrophages to become a major source of other pro-inflammatory cytokines and consistent with this previously described *in vitro* activity of fucoidans [280], we observed a marked reduction of most measured cytokines that originate from inflamed colon tissues.

Particularly TNF- α is thought to play a significant role in inflammatory cellular signalling, which is reflected by the successful clinical use of TNF- α inhibitors in patients with IBD [258]. Consistent with this hypothesis, macrophage-derived TNF- α production was reportedly blocked by fucoidan treatment in an *in vitro* co-culture model of gut inflammation [276,281]. However, in contrast to previous observations our results illustrate that the highly purified fucoidan extract (DPF) was unable to normalize the expression of TNF- α , while the polyphenol containing extract (*Synergy*) significantly reduced elevated TNF- α levels, which suggests additional benefits of the polyphenol content of fucoidan extracts to suppress TNF- α signalling.

In addition to macrophages, epithelial cells are also involved in driving the inflammatory cascade by secreting IL-6 in both patients and animal models of colitis [167]. Although our measurements for IL-6 under conditions of acute colitis exceeded the range of the assay, our data nevertheless suggest that both oral fucoidan extracts also reduce IL-6 levels (Fig. 4.4S), which would be consistent with previous observations [167].

One of the first studies of fucoidan use in a mouse model of acute colitis demonstrated reduced mucosal damage and retained crypt architecture [166]. In that study, in contrast to our approach of dietary treatment, fucoidan was administered intravenously. In line with this route of administration and results from *in vivo* microscopy, the authors convincingly argued that the beneficial effects were most likely explained by the well-known inhibition of selectins by fucoidans [151,156], which can prevent lymphocyte adhesion. In addition, fucoidans have also been reported to inhibit the next stage of lymphocyte tissue infiltration, the invasion, by directly inhibiting the expression [162] and activity of MMPs [156,282]. MMPs are implicated in tissue invasion of immune cells under pro-inflammatory conditions and, consistent with this inhibitory activity of fucoidans, histology results in our colitis model suggested significantly reduced lymphocyte invasion and oedema.

Given the significant size of fucoidans, which is between 5 and 1000 kDa, it appears plausible that the route of administration can significantly alter efficacy due to restricted absorption and tissue distribution of the parent molecule. Since oral fucoidans are likely to reach the target tissue in the case of IBD, most pre-clinical animal studies have therefore preferred oral formulations over intravenous injections for this indication [166,167,268]. Not only is this the most preferable form of administration for patients with IBD, it is also potentially the least stressful for rodents in pre-clinical *in vivo* studies. It nevertheless has to be stated that oral administration by gavage can also constitute a significant stress to the animals and if not performed accurately can be associated with damage to the GI tract. Consequently, previous studies used fucoidan-containing food chow to treat rodents, without having control over the ingested amount of fucoidan [167]. In contrast, the present study is the first to accurately control the amount of fucoidan in food that has been ingested per day by mixing a defined amount of fucoidan in a separate portion of food chow that the animals eat preferentially before eating their normal food. This method has been successfully used

before [273], is very well tolerated and also allows to study the unstressed behaviour of the animals due to minimal animal handling.

In line with previous reports, our results strongly support the use of oral fucoidan extracts while, in contrast, intraperitoneal fucoidan was unable to reduce disease severity in our disease model. In fact, in the case of body weight as a clinical marker, intraperitoneally administered fucoidan appeared to worsen the condition. This result was also reflected at the level of cytokines, where IPDPF showed a trend towards increasing the levels of some pro-inflammatory cytokines in DSS-treated animals. These results are not unexpected, since a recent study indicated that intraperitoneal fucoidan can effectively lead to the activation of dendritic cells (DCs) in the spleen [283]. The activation of DCs in this report was associated with increased production of IL-6, IL-12 and TNF- α . In line with an activation of immune cells by intraperitoneal fucoidan, we observed increased levels of IFN- γ and RANTES and a trend towards increased levels of IL-3, GM-CSF, IL-12(p40), IL-5 and IL-17 by IPDPF. In contrast to the study by Jin *et al.* [283], especially oral *Synergy* reduced the production of IL-12 and TNF- α , and likely also of IL-6, by cells of the intestinal tissue. These discrepant bioactivities highlight that the multiple activities of fucoidans are highly dependent on the specific formulation and route of administration used, which determines distribution to specific tissues in the organism. In line with several reports, our results do not provide any evidence for toxicity by oral fucoidan extracts [156,284,285].

Based on our *in vivo* findings, it forms an interesting picture that fucoidan extracts as well as enoxaparin could be potential therapeutic modalities for UC. We did not compare the efficacy of fucoidans to enoxaparin. Alternatively, it would be desirable to identify the active component(s) that (is) are responsible for the observed *in vivo* anti-inflammatory effects. Compared to enoxaparin, fucoidans are relatively more complex with a wider range

of MW (5 - 1000 kDa) and have different branching chains. In contrast, enoxaparin, a mixture of linear oligosaccharides, has a MW between 3800 to 5000 Da [51]. Different analytical techniques have been utilised to separate, isolate and elucidate the structural properties of these sulphated polysaccharides [131,172]. Commonly, the mixture is degraded enzymatically or chemically, before their structural analysis. Yet, the molecular structures including the chain length and sulphation pattern could be altered during depolymerisation process, complicating their structural characterisation. Currently there is a lack of standardised methods for fucoidan extraction as well as purification. Also, no efficient analytical techniques that are capable of separating the mixture of fucoidans to different components without changing the chemical and physical properties are available. In contrast, enoxaparin, a pharmaceutical grade anticoagulant agent, is produced from UFH using a well-established, standardised and quality-controlled method [49]. Unlike fucoidans, enoxaparin, without changing the chemical and physical properties, has been successfully separated into different oligosaccharides by IC technique according to their size and charge [76]. Hence, it would be interesting to identify the active component(s) which (is) are responsible for the observed anti-inflammatory activity of enoxaparin.

4.5 Conclusion

Overall, our results indicate that oral fucoidan extracts can significantly reduce the pathology associated with acute colitis induced by DSS. Given that multiple factors including disease location, type of inflammation, pathogenic mechanisms and the combination of multiple cytokines affect the response to colitis treatment [15], it is conceivable that drugs that target a single pro-inflammatory cytokine are likely to be limited in their ability to offer an effective maintenance therapy for IBD over extended periods of time. In contrast, fucoidans that simultaneously modulate multiple pro-inflammatory mechanisms and mediators could

provide a more sustainable strategy against intestinal inflammation. This study extends our current understanding of oral administration of fucoidan extracts during acute colitis, which is a crucial step towards its use for the treatment of colitis. The ability of fucoidan extracts to reduce inflammation and to retain epithelial integrity along with the possibility of oral delivery serves as justification to develop and evaluate fucoidan extracts as therapeutic alternatives for patients with IBD.

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Chapter 5

Chapter 6

Chapter 6 General Conclusion

6.1 Summary

Despite an increased understanding of the process involved in pathogenesis of UC, the exact cause of UC remains elusive. This debilitating disorder has no cure and the symptoms of colitis have a considerable impact on the quality of life. Currently approved therapeutic agents have limited efficacy and are associated with unwanted side effects. It highlights the need to develop new and safe therapeutic modalities for the management of UC. The naturally occurring highly sulphated polysaccharides, heparins and heparin-like molecules such as fucoidans, have received considerable interest as potential therapeutic options in UC.

Heparins (UFH and LMWHs) are clinically well-known anticoagulants. Their polysaccharide chains are heterogeneous both in their chemical compositions and chain lengths. Apart from their anticoagulant activity, there is evidence that heparins target multiple cellular events during inflammation. This has made heparins potentially important anti-inflammatory candidates. However, the current literature remains inconsistent with regards to the effects of heparins on cellular activities as well as the efficacy of heparins in UC. In addition, clinical applications of heparins in biological conditions other than where anticoagulation is required are largely hindered by their anticoagulant effects. This effect of heparins potentially increases the risk of bleeding. It is known that the pentasaccharide sequence in heparin chains is responsible for their anticoagulant activity. However, two third of heparin chains do not possess this sequence and may be responsible for other biological effects including anti-inflammatory effects. Thus we aimed to investigate the effects of heparins on cells and then identify oligosaccharides responsible for the effect of a LMWH, namely enoxaparin in colitis. Like heparins, fucoidans are also highly sulphated

polysaccharides and therefore have a wide range of biological activities including anti-inflammatory effects. Thus we also evaluated the potential of fucoidan extracts in colitis.

Using a number of cell-based assays, we first examined the effect of UFH, two LMWHs (namely dalteparin and enoxaparin) on colonic epithelial cell growth. Our results illustrated that UFH, enoxaparin and dalteparin inhibited cell proliferation. The inhibitory effect was well-correlated with the induction of cell cycle arrest in G1 phase and was not associated with significant changes in cell viability. Since enoxaparin was the most potent of them with regards to reduction of cell growth, we then tested the effect of individual oligosaccharides of enoxaparin. Enoxaparin was separated into different oligosaccharides using a validated IC method. The isolated oligosaccharides of intact enoxaparin have different degree of polymerisation ranging from 2 saccharides (dp2; ~ 600 Da) to 24 saccharides (dp24; ~ 8000 Da). Each individual 14 IC-derived oligosaccharides of enoxaparin had either no, anti- or pro-proliferative effects on cell growth, depending on its compositions. Disaccharide devoid of anticoagulant activity had the strongest anti-proliferative effect, whereas hexasaccharides actually promoted the cell growth.

Based on our *in vitro* results, we tested the effectiveness of enoxaparin using a pre-clinical model of colitis. Results obtained from an animal model of colitis indicated that orally administered but not intraperitoneal injected enoxaparin prevented the development of severe acute colitis. Oral treatment ameliorated colitis disease activity as well as alleviated the macroscopic and pathologic features of colons. It is conceivable that the observed effects of oral enoxaparin in mitigating acute colitis were associated, at least in part, with a reduction of macrophage-induced responses and the suppression of inflammatory cytokines.

Like enoxaparin, fucoidan extracts from *Fucus vesiculosus* when administered orally relieved acute colitis by reducing a number of inflammatory mediators. This effect was not

seen when fucoidans extracts were injected intraperitoneally. Similar to enoxaparin, given the nature of high complexity, it is unclear which active components in the mixtures of fucoidan polysaccharides contribute to the observed activity. Both enoxaparin and fucoidans are polydisperse mixture of oligosaccharides or polysaccharides of variable molecular weights. Which oligosaccharide(s) from the mixture is (are) responsible for their effect in colitis is unknown at present. Hence, it is difficult to elucidate the associated mechanism of enoxaparin and fucoidan extracts in reducing colitis. We therefore tested different oligosaccharides of enoxaparin in order to identify the oligosaccharide(s) responsible for the effect of intact enoxaparin. The results indicated that oligosaccharides (dp2 - dp6) shorter than dp8 were responsible for the activity of enoxaparin. Among the oligosaccharides tested, tetrasaccharide and hexasaccharide were found the most active in preventing colitis-associated body weight loss and colonic changes.

Our *in vitro* and *in vivo* results indicated that enoxaparin oligosaccharides could affect cellular response as well as colitis, depending on the composition of oligosaccharides. Currently, all commercially available heparins are standardised according to their anti-factor Xa activity and are not tested for their non-anticoagulant activities. Our data for the first time can explain the inconsistent biological effects of heparins when different preparations were investigated for the biological effects other than anticoagulant activity. Depending on the structural compositions, different heparin preparations could result in different biological responses. Although the structures of oligosaccharides required for the activity have not been identified, the results demonstrated that the non-anticoagulant activities of enoxaparin including the anti-colitis effect are unrelated to the antithrombin binding pentasaccharide. In fact, disaccharide and tetrasaccharide are insufficient in length to bind antithrombin for the expression of anticoagulant effect, thus eliminating the risk of bleeding.

In summary, highly sulphated polysaccharides including enoxaparin and fucoidan extracts have anti-inflammatory potential in colitis. Specifically, enoxaparin relieved inflammation by reducing macrophage-associated inflammatory responses. Oral administration is likely to deliver these anionic macromolecules directly to the sites of inflammation, to exert local effects. This route of administration is especially convenient when used for managing UC. Furthermore, isolating and testing different oligosaccharides of enoxaparin allowed us to identify specific oligosaccharides that are responsible for the observed biological effects. Our approach highlighted the importance of evaluation of the biological effects attributed by different oligosaccharides present in enoxaparin or other mixtures of sulphated polysaccharides, as a first step toward novel oligosaccharide-based therapies.

6.2 Limitations

This study evaluated sulphated polysaccharides including heparins and fucoidans via cell-based assays and in a model of colitis. As a direct consequence of the methodology used, a number of limitations need to be considered. The nature of *in vitro* assays is the absence of internal biochemical processes and the inability to represent complex interaction or responses in organism. It is unclear how heparins and the oligosaccharides of enoxaparin affect cell growth, resulting in either inhibition or stimulation of cell proliferation.

In this study, we used a mouse model of DSS-induced acute colitis. DSS-induced colitis is a robust and low cost screening method to identify potential therapeutic candidates. However, the efficacy of enoxaparin, its oligosaccharides and fucoidans extracts administered during colitis induction did not address the potential of these agents in treating colitis. We opted for 8-days prophylactic protocol instead of treatment after colitis induction due to the ethical concern in our setting (> 15% of body weight loss required euthanasia) as

well as to limit the discomfort and the pain caused to the animals. To increase the holding capacity of animal facility, there were inevitable changes in animal housing system, which resulted in alteration of colitis disease activity induced by DSS.

In the animal study, we only tested the effect of a single LMWH (enoxaparin), its oligosaccharides and two fucoidan extracts, both derived from *Fucus vesiculosus* at the selected doses. The optimal dose of each agent was not determined. The effects of UFH, other LMWHs as well as fucoidans obtained from other algal species were not investigated. Since no two preparations of UFH, LMWHs or fucoidan extracts from different species are similar in their oligosaccharide composition, the results of this study cannot be generalised to other types of heparins or fucoidan extracts. Although the IC technique enabled the separation of enoxaparin into sufficient amount of intact oligosaccharides for *in vitro* and *in vivo* study, the scalability of this method is limited by the use of semi-preparative CarboPac PA100 column.

6.3 Future directions

Finding new therapeutics to manage chronic inflammatory diseases is not only important but also challenging. It will be interesting to investigate other heparins or heparin-like molecules to see whether these highly sulphated polysaccharides share similar properties or they behave differently depending on their unique compositions. In addition, the development of sulphated polysaccharide-based therapies requires robust testing and a thorough understanding of their structure-activity relationships. A combination of orthogonal analytical approaches is required to permit structural analysis of the oligosaccharide for the identification of their composition, sequence, active motive and molecular target with a view to relate this information to their observed activity. Animal models of chronic colitis which simulate human UC could be further used to confirm the

effect of fucoidan extracts, enoxaparin and its oligosaccharides as well as to investigate the potential mechanism (s) by which they exert the anti-inflammatory activity. Future studies should also aim to identify the optimal doses as well as the safety of enoxaparin oligosaccharides when used in patients with UC.

There is a need to advance the separation technology to scale up enoxaparin oligosaccharides for the formulation of desired dosage forms. Also, using well-designed animal models, the pharmacokinetic profiles and optimal dose ranges of oligosaccharides for anti-UC effect should be determined. Since the absorption of oral heparin oligosaccharides through intestinal mucosa is poor due to their lack of lipophilicity and large size, the orally administered oligosaccharide can be selectively delivered to the colonic site of inflammation in UC. Oral targeted- released formulation improves patient compliance due to ease of administration and minimal side effects.

To develop this innovative treatment more efficiently, another important aspect is to ensure the product meets the regulatory standards in terms of stability, safety, quality control and assurance. Reliable assays should be developed to compare and standardise the properties and thus the anti-UC activities of oligosaccharides. For example, molecular weight properties of oligosaccharides, which are known to impact their biological activity, should meet the range of molecular weight restrictions to minimize variation within commercially derived heparin oligosaccharides. All the results that will be obtained through the approach suggested here can form the basis for the development of heparin-derived oligosaccharides for the treatment of UC. Unlike investigations using a novel drug moiety, the vast clinical experience with heparins would significantly facilitate a rapid bench-to-bedside transition.

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Appendices

Chapter 8 Appendices

8.1 Appendix A

Ethics Approval from Animal Ethics Committee of University of Tasmania

	University of Tasmania Animal Ethics Committee ETHICS APPROVAL PERMIT	University of Tasmania Office of Research Services Ph: 03 62267283 Fax: 03 62267148 animal.ethics@utas.edu.au
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To: Dr Raj Eri
From: Marilyn Pugsley Executive Officer Animal Ethics
Date: 23 October 2013
Project: **A13576 – The potential of enoxaparin in ulcerative colitis**
Approved on: 23 October 2013
Approval expires: 23 October 2016
1st Annual Report due: 23 October 2014

Please read this permit carefully as **approval may be withdrawn**
for projects that do not comply with the conditions

The Animal Ethics Committee has approved the above project. The approval is subject to the review and approval of an annual report which is due on the approval anniversary. **Please note this date in your diary.**

If the project is to continue past the expiry date, a new initial application will need to be submitted. A project can only be approved for a maximum of 3 years.

As the Responsible Investigator, you **MUST** ensure that:

- (a) all aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013
- (b) a full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.
- (c) you contact the Animal Welfare Officer, Dr Sue Ottomanski (sue.ottomanski@utas.edu.au) to advise her when and where your experiments will be conducted. Sufficient notice needs to be given so that if the AWO wishes to make an inspection, this can be easily arranged.
- (d) That all investigators attend Ethics training sessions every three years. Contact the Executive Officer Animal Ethics for the next available session.

The Animal Ethics Committee is to be promptly notified of any unexpected events which occur during the period of the approved project and impact on the welfare of the animals.

Autopsy should be performed by a qualified veterinarian when animals die unexpectedly. Any foreseeable departure from this requirement must have been outlined and approved in the initial application.

Animals approved:

Mice C57BL/6 – 230 total

Marilyn Pugsley
Executive Officer Animal Ethics

8.2 Appendix B

Slide no:

Histology score sheet for DSS-induced acute colitis

	PC	DC
Distribution of inflammation		
0=none		
1= focal		
2=multifocal		
3=diffuse		
Crypt Architecture		
0=normal		
1=irregular		
2=moderate crypt loss (10-50%)		
3=severe crypt loss (50-90%)		
4=small/medium sized ulcers (<10 crypt widths)		
5=large ulcers (>10 crypt widths)		
Loss of crypts		
0=no damage		
1=30% shortening of crypts		
2=65% shortening of crypts		
3=total loss of crypts		
4=loss of entire epithelial layer		
Extent of crypt loss in the region		
0=no crypt loss		
1=<25%		
2=25-50%		
3=50-75%		
4≥75%		
Tissue Damage		
0=no damage		
1=discrete lesions		
2=mucosal erosions		
3=extensive mucosal erosion with submucosal damage		
Goblet Cell Loss		
0= normal <10% loss		
1=10-25%		
2=25-50%		
3≥50%		
Submucosa scored for oedema		
0 = no change		
1 = mild		
2 = moderate		
3 = profound		
Inflammatory Cell Infiltration		
0=occasional infiltration		
1=increasing leukocytes in lamina propria		
2=confluence of leukocytes extending to submucosa		
3=transmural extension of inflammatory infiltrates		
Crypt abscesses		
0=none		
1=1-5		
2=6-10		
3≥10		